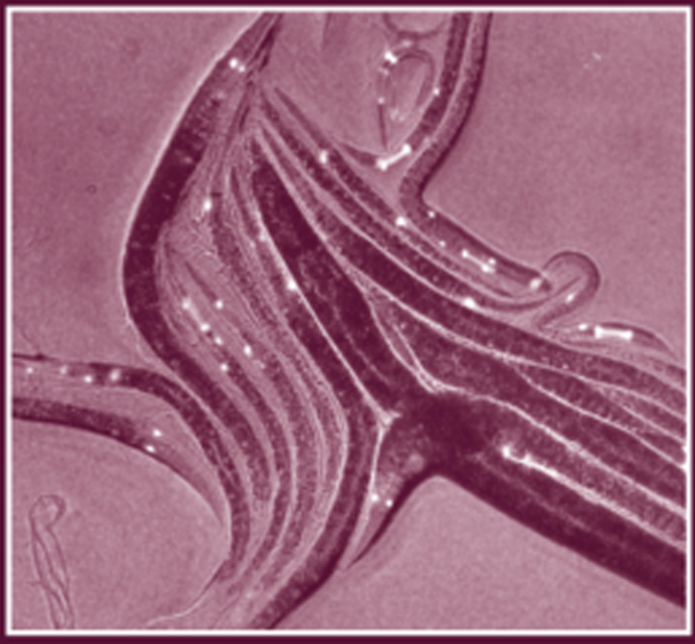
# INTERNATIONAL REVIEW OF CYTOLOGY

## A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



Volume 209

ACADEMIC PRESS

# International Review of Cytology
## A Survey of Cell Biology

VOLUME 209

# International Review of Cytology
# A Survey of Cell Biology

Edited by

**Kwang W. Jeon**
Department of Biochemistry
University of Tennessee
Knoxville, Tennessee

**VOLUME 209**

ACADEMIC PRESS
A Harcourt Science and Technology Company
San Diego San Francisco New York Boston London Sydney Tokyo

*Front cover photograph:* Effect of deletion of block D on the expression of *ace-1::*GFP. (See Chapter 4, figure 7 for more details.)

This book is printed on acid-free paper. ♾



Academic Press
*A Harcourt Science and Technology Company*
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.academicpress.com

Academic Press
Harcourt Place, 32 Jamestown Road, London NW1 7BY, UK
http://www.academicpress.com

International Standard Book Number: 0-12-364613-8

PRINTED IN THE UNITED STATES OF AMERICA
01 02 03 04 05 06 EB 9 8 7 6 5 4 3 2 1

# CONTENTS

## The Biology of Cortical Granules

Gary M. Wessel, Jacqueline M. Brooks, Emma Green, Sheila Haley, Ekaterina Voronina, Julian Wong, Victor Zaydfudim, and Sean Conner



## Acetylcholinesterase Genes in the Nematode *Caenorhabditis elegans*

Didier Combes, Yann Fedon, Jean-Pierre Toutant, and Martine Arpagaus



## Reciprocal Regulation of Endothelin-1 and Nitric Oxide: Relevance in the Physiology and Pathology of the Cardiovascular System

Gian Paolo Rossi, Teresa Maria Seccia, and Gastone G. Nussdorfer

This Page Intentionally Left Blank

# CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.


Martine Arpagaus (207), *Différenciation Cellulaire et Croissance/INRA, 34060 Montpellier Cedex 1, France*

Jacqueline M. Brooks (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Guojun Bu (79), *Department of Pediatrics and of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110*

Didier Combes (207), *Différenciation Cellulaire et Croissance/INRA, 34060 Montpellier Cedex 1, France*

Sean Conner (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Yann Fedon (207), *Différenciation Cellulaire et Croissance/INRA, 34060 Montpellier Cedex 1, France*

Emma Green (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Sheila Haley (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Malcolm Maden (1), *MRC Centre for Developmental Neurobiology, King's College, London SE1 1UL, United Kingdom*

Gastone G. Nussdorfer (241), *Department of Human Anatomy and Physiology, University of Padua, I-35121 Padova, Italy*

Gian Paolo Rossi (241), *Department of Clinical and Experimental Medicine, University Hospital, I-35121 Padova, Italy*

Teresa Maria Seccia (241), *Department of Clinical and Experimental Medicine, University Hospital, I-35121 Padova, Italy*

Jean-Pierre Toutant (207), *Différenciation Cellulaire et Croissance/INRA, 34060 Montpellier Cedex 1, France*

Ekaterina Voronina (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Gary M. Wessel (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Julian Wong (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Victor Zaydfudim (117), *Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

# Role and Distribution of Retinoic Acid during CNS Development

Malcolm Maden
MRC Centre for Developmental Neurobiology, King's College London
London SE1 1UL, United Kingdom

Retinoic acid (RA), the biologically active derivative of vitamin A, induces a variety of embryonal carcinoma and neuroblastoma cell lines to differentiate into neurons. The molecular events underlying this process are reviewed with a view to determining whether these data can lead to a better understanding of the normal process of neuronal differentiation during development. Several transcription factors, intracellular signaling molecules, cytoplasmic proteins, and extracellular molecules are shown to be necessary and sufficient for RA-induced differentiation. The evidence that RA is an endogenous component of the developing central nervous system (CNS) is then reviewed, data which include high-pressure liquid chromotography (HPLC) measurements, reporter systems and the distribution of the enzymes that synthesize RA. The latter is particularly relevant to whether RA signals in a paracrine fashion on adjacent tissues or whether it acts in an autocrine manner on cells that synthesize it. It seems that a paracrine system may operate to begin early patterning events within the developing CNS from adjacent somites and later within the CNS itself to induce subsets of neurons. The distribution of retinoid-binding proteins, retinoid receptors, and RA-synthesizing enzymes is described as well as the effects of knockouts of these genes. Finally, the effects of a deficiency and an excess of RA on the developing CNS are described from the point of view of patterning the CNS, where it seems that the hindbrain is the most susceptible part of the CNS to altered levels of RA or RA receptors and also from the point of view of neuronal differentiation where, as in the case of embryonal carcinoma (EC) cells, RA promotes neuronal differentiation. The crucial roles played by certain genes, particularly the *Hox* genes in RA-induced patterning processes, are also emphasized.

**KEY WORDS:** Retinoic acid, Retinoid receptors, Central nervous system, Neuronal differentiation, *Hox* genes. 

## I. Introduction

Retinoic acid (RA) is a low molecular weight (approximately 300) lipophilic compound derived from vitamin A which acts at the level of the nucleus to establish or maintain patterns of gene activity. Vitamin A, or retinol, is obtained from the diet in the form of carotenoids (from plant sources) or retinyl esters (from animal sources) and the family of molecules derived from retinol is known as the retinoids. During development, therefore, mammalian embryos obtain their retinoids from the maternal circulation via the placenta, whereas bird, reptilian, amphibian, and fish embryos obtain their retinoids from stores in the yolk. In the adult it has long been known that retinoids are required for vision, the maintenance of the differentiated state of the skin, spermatogenesis, and the maintenance of the blood and immune systems, and in the embryo that retinoids are crucial for the development of various organ systems. The most recent data have demonstrated that retinoids are required for the development of the central nervous system (CNS) and this is the subject of this review.

Cells that require RA metabolize it from retinol, which arrives in the blood bound to retinol-binding protein, having been released from the liver stores (Vogel *et al.,* 1999). This conversion to RA is accomplished within the cell by the action of two types of enzyme. The first type of enzyme, the retinol dehydrogenases or alcohol dehydrogenases, convert retinol into retinaldehyde and then the second type, the retinal dehydrogenases, convert retinaldehyde into RA (Blaner *et al.,* 1999). There are several different forms of RA: all-*trans*-RA and 9- *cis*-RA, which are the ligands for the nuclear retinoic acid receptors (see below); didehydroRA, which is found predominantly in bird embryos rather than mammalian embryos; and 4-*oxo*-RA, 4-OH-RA, and 18-OH-RA, which are generated from all-*trans* and 9-*cis*-RA by a cytochrome *P*-450 enzyme called CYP26.

All-*trans*-RA and 9-*cis*-RA act within the nucleus to establish or change the pattern of gene activity in cells. This occurs because of the existence of ligand activated transcription factors within the nuclei of RA-sensitive cells. There are two classes of these transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), and they form part of the gene superfamily including the steroid hormone receptors. In human and mouse, three RARs have been identified and designated RAR$\alpha$, RAR$\beta$, and RAR$\gamma$ (Kastner *et al.,* 1994b). There are also three RXRs: RXR$\alpha$, RXR$\beta$, and RXR$\gamma$ (Kliewer *et al.,* 1994). Each of these six receptors is encoded by a specific gene from which multiple isoforms can be generated by differential splicing and multiple promoters. The ligand for the RXRs is 9-*cis*-RA, whereas the RARs bind both 9-*cis*-RA and all-*trans*-RA. These receptors act as ligand-dependent transcription factors by recognizing consensus sequences known as retinoic acid response elements (RAREs), which are present in the enhancer sequences of RA- responsive genes. The RARs and RXRs do not act alone, but as heterodimers, and the RXRs can also heterodimerize with a variety

of other related receptors such as the thyroid hormone receptors, the vitamin D receptors, the peroxisome proliferator-activated receptors (PPARs, whose ligands are fatty acids), LXRs (whose ligands are oxysterols), FXRs (whose ligands are farnesoids), or other orphan receptors such as NGFIB (which is a nerve growth factor-induced receptor). These analyses thus reveal how retinoids can elicit such a diversity of biological responses.

This review begins by describing the molecular events involved when RA induces undifferentiated cell lines to turn into neurons and glia. These observations are highly suggestive of a role for RA in the developing CNS, but more definitive evidence has come from various sources: detecting endogenous retinoids within the developing CNS; determining the distribution of the retinoic acid synthesizing enzymes; determining the distribution of the retinoid-binding proteins and the retinoic acid receptors; knocking out or interfering with the signaling functions of the receptors; and studying the effects of an excess or a deficiency of retinoids on the developing CNS. Each of these topics will be reviewed here, beginning with the induction of neuronal differentiation in various undifferentiated cell types by RA.

## II. The Induction of Neuronal Differentiation in Culture

### A. Embryonal Carcinoma Cells and Neuroblastoma Cells

Amazingly, when RA is added to embryonal carcinoma (EC) cells, the type of differentiated cell induced depends on the concentration of RA applied. At low doses cardiac muscle is induced, at intermediate doses skeletal muscle is induced, and at high doses neurons and astroglia are induced (Edwards and McBurney, 1983). In fact, at the doses of RA required to induce neural genes, the genes involved in mesodermal differentiation such as actin are actively repressed (Bain *et al.,* 1996), so the mechanics of neural differentiation by RA must involve the induction of both activators and repressors. Many of the initial observations were made using the P19 strain of EC cells and it was also shown to be the case in several other EC strains, stem cells, teratocarcinoma cells, or neuroblastoma cells derived from the mouse (Jones-Villeneuve *et al.,* 1982; Kuff and Fewell, 1980; Liesi *et al.,* 1983; McBurney *et al.,* 1982) or the human (Sidell, 1982; Andrews, 1984; Thompson *et al.,* 1984).

There are several reasons why these observations on cultured cells are important. The first reason is an embryological one: if different concentrations of RA induce different cell types, then it is possible that in the embryo different concentrations of endogenous RA might be responsible for the induction of the different parts of the embryo such as the CNS, the mesoderm, or the heart. The second reason is a clinical one: because human neuroblastoma cells can be induced to differentiate

into neurons by RA (Pahlman *et al.,* 1984; Shea *et al.,* 1985; Sidell, 1982; Sidell *et al.,* 1983), then one may be able to use RA as a therapeutic treatment for neuroblastomas *in vivo*. The third reason is also a clinical one: because EC cells and human neuroblastoma cells can be differentiated into neural cells they can then be used as transplanted tissue to either promote recovery from ischemic damage (Borlongan *et al.,* 1998) or to act as a long-term depot for the slow release of neuropeptides (Pleasure *et al.,* 1992) or other gene products of therapeutic value for example in the treatment of Parkinson's disease (Nakao *et al.,* 2000) or Alzheimer's disease (Trojanowski *et al.,* 1997). RA induces the dopaminergic phenotype in human neuroblastoma cells (Farooqui, 1994) so perhaps they could be used as grafts in the treatment of Parkinson's, thereby obviating the ethical debate on the use of embryonic tissues. RA is also used to differentiate adult CNS stem cells derived from the striatum or hippocampus (Takahashi *et al.,* 1999; Wohl and Weiss, 1998) and such stem cells also have remarkable potential for therapeutic use.

Since the original observations of the induction of neuronal differentiation by RA in EC cells, this experimental paradigm has become the mainstay for discovering the downstream targets that RA switches on in order to induce the appearance of neurites and for testing the role of particular genes/proteins in the neural differentiation process. Clearly, it is easier to perform such experiments on cultured cells rather than whole animals because of the quantities of relatively pure populations of cells available and their accessibility to alteration. Many known genes have been examined, for example, 23 markers were used in a study using human neuroblastoma cells by Hill and Robertson (1997) and many novel genes have been discovered by differential screening between normal and RA treated cells (e.g., Jonk *et al.,* 1994; Bouillet *et al.,* 1995a; Wakeman *et al.,* 1997; Cheung *et al.,* 1997), attesting to the usefulness of the cell culture system. Most recently, cDNA arrays have been used to detect 76 up-regulated genes and 11 down-regulated genes (Satoh and Kuroda, 2000). The genes/proteins described and discovered include transcription factors, structural proteins, enzymes, cell surface glycoproteins, extracellular proteins, neurotransmitters, neuropeptide hormones, growth factors, and their receptors, as will now be elaborated.

## 1. Nuclear Events

We might expect that since neurons are induced by RA treatment of cell lines then neuronal determination transcription factors would be induced and indeed this is the case. The mammalian homolog of the *Drosophila* neural determination gene *achete-scute,* MASH1, is induced to high levels whereas MASH2 is repressed (Johnson *et al.,* 1992; Bain *et al.,* 1996). Curiously, another transcription factor, myocyte enhancer factor 2 (MEF2), is also induced coordinately with MASH1 in P19 cells despite being involved in muscle differentiation (Mao and Nadal-Ginard, 1996; Black *et al.,* 1996). A physical interaction occurs between MASH1

and MEF2 in the nucleus such that MASH1/MEF2 heterodimers induce neurons, whereas myogenic transcription factors/MEF2 heterodimers induce muscle.

The homeobox genes *Hoxa-7, Hoxb-5,* and *En-1* are induced (Deschamps *et al.,* 1987a, 1987b) as is *Pax-3* (Goulding *et al.,* 1991), *Pax-6* (Gajovic *et al.,* 1997), and several other homeodomain proteins: *Pbx1* (Knoepfler and Kamps, 1997), *Meis2* (Oulad-Abdelghani *et al.,* 1997), *Gbx-2* (Bouillet *et al.,* 1995b), and *Ptx2* (Lindberg *et al.,* 1998). Other transcription factors induced include ATBF1 (Ido *et al.,* 1994), AP-2 (Phillip *et al.,* 1994; Ohkawa *et al.,* 1999), a RING finger protein called BFP (Inoue *et al.,* 1997), *Hnf3α* and *Hnf3β* (Jacob *et al.,* 1997), and a neural cell nuclear factor related to the retinoic acid receptors (Bauer *et al.,* 1997). On the other hand, some are down-regulated including a zinc finger gene, *zf5-3* (Dimitroulakos *et al.,* 1999) and a POU domain gene, *tai-ji* (Huang and Sato, 1998).

Of course, it is not necessarily the case that since a transcription factor is induced by RA it is essential for neural induction, but in four cases at least, their causal role has been demonstrated. *Brn-2* is a POU domain transcription factor that is CNS specific, and RA treatment of P19 cells induces this molecule (Fujii and Hamada, 1993), whereas *Brn-3.2* is down-regulated by RA (Turner *et al.,* 1994). When the cells were transfected with a vector expressing antisense *Brn-2* then RA-induced neuronal differentiation was blocked and instead the cells differentiated into muscle (Fujii and Hamada, 1993). Furthermore, one of the downstream targets of *Brn-2* is corticotropin-releasing hormone (CRH) and this neuropeptide also fails to be induced after RA treatment of neuroblastoma cells when they have been transfected with antisense *Brn-2* (Ramkumar and Adler, 1999). Similarly, NF-kB is a transcription factor that is induced in SH-SY5Y neuroblastoma cells after RA treatment, following which the cells differentiate along the chromaffin lineage. When the dominant negative form of NF-kB is overexpressed, then there is no neuronal differentiation after RA (Feng and Porter, 1999). *Stra13* is a basic helix–loop–helix transcription factor that was identified in a differential screen between normal and RA treated P19 cells. When overexpressed in P19 cells this gene promotes neural differentiation (Boudjelal *et al.,* 1997). Finally, SOX1 is one of the earliest transcription factors to be expressed in ectodermal cells committed to the neural fate in the embryo. RA induces SOX1 in P19 cells during neuronal differentiation and when SOX1 overexpressing cell lines were created they expressed *Nestin, Mash1, Hoxa-7,* and neurofilament proteins and mature neurons appeared as well as GFAP positive glial cells, thereby overcoming the requirement for RA (Pevny *et al.,* 1998)

There are also changes in the nuclear structural proteins, the lamins (Pierce *et al.,* 1999) and other nuclear matrix proteins (Kim *et al.,* 1998), and a novel nuclear protein called necdin is induced in P19 cells (Marayama *et al.,* 1996). In addition, the gene for an RNA binding protein called HUD has been cloned from P19 and PC12 cells and it is found to be expressed in the Purkinje cells of the cerebellum (Steller *et al.,* 1996).

Because proliferation is inhibited as the undifferentiated cells differentiate into neurons, changes occur in cell cycle control genes. E2F is a transcription factor induced in the cell cycle and it is repressed (Gill *et al.,* 1998) as are the N-*myc* and the c-*myb* genes (Amatruda *et al.,* 1985; Thiele *et al.,* 1985, 1988; Tonini *et al.,* 1999) whereas the *src* gene is up-regulated (Lynch *et al.,* 1986). The retinoblastoma protein is induced and becomes phosphorylated by Cdk4 kinase activity and other cyclins (Gill *et al.,* 1998; Watanabe *et al.,* 1999). An additional nuclear gene, a member of the nucleosome assembly proteins HB20 is induced (Fan *et al.,* 1998). Another gene involved in growth suppression, called *DAN,* is induced in neuroblastoma cells (Nakamura *et al.,* 1998). DAN seems to play a role in differentiation because its overexpression potentiates the effects of RA in making longer neurites, which appear earlier during the differentiation process.

One would also expect the RARs to be involved in transducing the RA signal to differentiate. Thus in neuroblastoma cells and mouse EC cells there is an up-regulation in RAR$\beta_2$ and RAR$\alpha$, specifically RAR$\alpha_2$ (Heiermann *et al.,* 1992; Wuarin *et al.,* 1994) and a decrease in RAR$\gamma_1$ (Ferrari *et al.,* 1998). RAR$\gamma_1$ seems to be a repressor of RAR$\beta_2$ and they are both important regulatory events because if RAR$\gamma_1$ is transfected into cells the response to RA is decreased (Cheung *et al.,* 1997; Ferrari *et al.,* 1998). Similarly, if RAR$\beta$ is transfected into cells more of them produce neurites in response to RA (Cheung *et al.,* 1997). In addition, in a mutant mouse EC cell line that does not differentiate in response to RA, none of these alterations in RARs occurs (Heiermann *et al.,* 1992). In another nonresponding mutant P19 cell line, RAC65, the RAR$\alpha$ gene is truncated and acts as a dominant-negative repressor (Pratt *et al.,* 1990). However, this is not the only cause of the loss of responsiveness because transfecting in the mutant RAR$\alpha$ into normal cells does not confer nonresponsiveness to RA. It now appears that this mutant cell line responds perfectly to 9-*cis*-RA, implying that the loss of responsiveness is due to a defect in the RXRs, possibly RXR$\gamma$ (Yokota and Ohkubo, 1996). There seem to be very few reports of any effects of RA on the RXRs, but one such example is in the induction of RAR$\alpha$ and RXR$\alpha$ in human medulloblastoma cells (Wang and Christakos, 1995). However, the use of RAR and RXR selective agonists has revealed that although RXR agonists are ineffective at inducing neurofilament positive neurons, they synergize with RAR agonists, suggesting that a RAR/RXR heterodimer (either the RAR$\alpha$/RXR or RAR$\gamma$/RXR heterodimer) is the effective receptor combination transducing the differentiation signal (Roy *et al.,* 1995; Horn *et al.,* 1996).

Curiously, it is a routine observation that apoptosis occurs in a proportion of cells during RA-induced differentiation (Castro-Obregon and Covarrubias, 1996; Okazawa *et al.,* 1996; Horn *et al.,* 1996; Ninomiya *et al.,* 1997; Herget *et al.,* 1998). Thus we can classify the types of differentiated products that occur after RA treatment as nerves, astrocytes (and oligodendrocytes), fibroblast-like cells, and apoptotic cells. Apoptosis decreases with time as differentiation sets in due to the induction of *Bcl-2* and the down-regulation of *Bax* and *Bak* (Lasorella

*et al.,* 1995; Herget *et al.,* 1998; Wang and Halvorsen, 1998). 9-*cis*-RA is more effective than all-*trans*-RA at inducing apoptosis in P19 cells, and when RAR and RXR selective agonists were used they showed synergistic effects suggesting that RAR/RXR heterodimers were involved in transducing the RA signal (Horn *et al.,* 1996). These results confirm that apoptosis is a specific receptor-mediated event that is a part of the differentiation program and not just a result of treating cells with a toxic compound.

## 2. Cytoplasmic Events

As might be expected for neuronal differentiation, the major cytoplasmic changes seem to involve cytoskeletal proteins. Thus the microtubule-associated proteins MAP1A, MAP1B, MAP2, and tau are induced (Pleasure *et al.,* 1992; Fischer *et al.,* 1985; Vaillant and Brewin, 1995; Fraichard *et al.,* 1995), as is $\beta$-tubulin (Bain *et al.,* 1995; Vaillant and Brewin, 1995), GAP-43 (Finley *et al.,* 1996; Pleasure *et al.,* 1992; Esdar *et al.,* 1999; Hill and Robertson, 1997), along with the ubiquitous finding of the induction of neurofilament proteins (e.g., Paterno *et al.,* 1997). Further cytoskeletal proteins such as neuronal thread proteins are induced (de la Monte *et al.,* 1996) and a member of an actin-binding family of genes, ENC1, is up-regulated (Hernandez *et al.,* 1998). Others, presumably associated with the undifferentiated state, such as cytokeratin and vimentin, are down-regulated (Hill and Robertson, 1997; Bani-Yaghoub *et al.,* 1999). Interestingly, in cells transfected with antisense MAP2, RNA neuronal differentiation can no longer be induced by RA (Dinsmore and Solomon, 1991), thus attesting to the essential role of these cytoskeletal proteins in RA-induced differentiation.

The components of the intracellular signaling cascade are induced. Myoinositol concentrations increase inside the cell (Novak *et al.,* 1999) and the inositol triphosphate receptor $IP_3R1$ is induced (Ohkawa *et al.,* 1999). Other work has, however, reported a decrease in myoinositol phospholipid metabolism reflected in a decrease in inositol triphosphate and diacylglycerol content (Ponzoni and Lanciotti, 1990). Ras is activated (Tonini *et al.,* 1999), G proteins are induced (Morishita *et al.,* 1999), calcium-dependent phophorylation events take place (Mizuno *et al.,* 1989), and specific protein kinase C (PKC) isoforms are induced (Oehrlein *et al.,* 1998). The importance of PKC$\varepsilon$ in the gene cascade leading to the appearance of neurons has been demonstrated by overexpressing it in neuroblastoma cells, which results in the induction of neurites (Zeidman *et al.,* 1999). Adenylate kinases and adenylyl cyclases are also induced (Inouye *et al.,* 1998; Lipskaia *et al.,* 1997) and the activity of the calcium/calmodulin-dependent protein kinase II promoter is stimulated by RA (Chen and Kelley, 1996). On the other hand, a protein tyrosine phosphatase, SHP-1, is down-regulated, and when this gene is overexpressed in P19 cells the cells do not differentiate after RA treatment (Mizuno *et al.,* 1997). The effects must be different on different protein tyrosine phosphatases because in mouse embryonic stem cells the levels of protein tyrosine phosphatase $\alpha$ is increased by RA and

when cells are transfected with this gene it enhances the effect of RA (van Inzen *et al.,* 1996). Ceramide, a component of the sphingomyelin pathway that responds to extracellular signals, is induced in mouse neuroblastoma cells and the addition of ceramide alone to these cells induced neurite outgrowth (Riboni *et al.,* 1995).

A phosphoprotein gene is induced, called *hUlip,* in neuroblastoma cells that has a homolog in *Caenorhabditis elegans* (Gaetano *et al.,* 1997). Animals that are mutant in this gene have defects in axonal outgrowth and have uncoordinated movements, suggesting it is playing an important role.

Enzymes that generate neurotransmitters from each of the major classes are induced: choline acetyl transferase and acetylcholine esterase of the cholinergic neurons (Sharma and Notter, 1988; Bussiere *et al.,* 1995; Fraichard *et al.,* 1995; Parnas and Linial, 1995; Hill and Robertson, 1997; Coleman and Taylor, 1996; Renoncourt *et al.,* 1998), glutamic acid decarboxylase of the GABAergic neurons (Bain *et al.,* 1996; Fraichard *et al.,* 1995; Renoncourt *et al.,* 1998), tyrosine hydroxylase of the dopaminergic neurons (Cosgaya *et al.,* 1996; Renoncourt *et al.,* 1998), and various neuropeptide transmitters (Hill and Robertson, 1997). A neuroendocrine specific protein is induced (Hens *et al.,* 1998) and corticotropin-releasing factor (CRF) is synthesized in neuroblastoma cells (Kasckow *et al.,* 1994). The synthesis of CRF is prevented if the up-regulation of transcription factor *Brn-2* is prevented, thus demonstrating a link between a transcription factor and a neuropeptide (Ramkumar and Adler, 1999). Another enzyme induced is nitric oxide synthase (Ogura *et al.,* 1996). A calcium binding protein, calbindin, is induced in two human medulloblastoma cell lines by RA (Wang and Christakos, 1995).

Presenilin is an intracellular membrane protein that may cleave the amyloid precursor protein. It has assumed a great significance since mutations in the presenilin gene are responsible for early-onset Alzheimer's disease. In human neuroblastoma cells the amyloid precursor protein gene itself is up-regulated by RA (Konig *et al.,* 1990) and in P19 cells both presenilin 1 and presenilin 2 are induced during RA-induced neuronal differentiation (Kawamura *et al.,* 1999; Cullenor *et al.,* 2000), and in human teratocarcinoma cells only presenilin 1 is induced (Ren *et al.,* 1999). The amyloid precursor protein (APP) itself is induced in P19 cells (Hung *et al.,* 1992; Cullenor *et al.,* 2000) as well as a molecule that interacts with presenilin 1 and is a member of the armadillo family (Kawamura *et al.,* 1999). Both presenilin 1 and APP seem to be important molecules on the neuronal differentiation pathway because when human teratocarcinoma cells are transfected with presenilin 1 antisense cDNA they fail to differentiate after RA treatment and there is no similar effect with presenilin 2 (Hong *et al.,* 1999). Furthermore, when these cells are transfected with the mutant form of presenilin 1 that is found in early-onset Alzheimer's disease, the induction of neurons by RA is dramatically attenuated (Tokuhiro *et al.,* 1998). Similarly, when neuroblastoma cells are transfected with the C-terminal peptide of APP, RA-induced neuronal differentiation is inhibited (Honda *et al.,* 1998).

## 3. Cell Surface and Receptor Events

The differentiation of F9 cells into neurons is accompanied by a change in cell surface glycolipids (Andrews *et al.,* 1986), there is a change in the synthesis of gangliosides (Osani *et al.,* 1997), and more complex gangliosides appear (Levine and Flynn, 1986). Ten ganglioside induced differentiation proteins are induced (Liu *et al.,* 1999). However, if ganglioside synthesis is inhibited, RA still induces neurites so this cannot be an obligatory step in the RA pathway (Li and Ladish, 1997). More specifically, certain molecules and enzymes have now been identified such as the RA-induced glycosylation of alkaline phosphatase (Mueller *et al.,* 2000), specific sialyltransferases such as GD3 synthase (Osani *et al.,* 1997) and the altered activities of a specific glycosyltransferase, UDP-*N*-acetylglycosamine:dolichylphosphate *N*-acetylglucose-1-phosphate transferase (Meissner *et al.,* 1999). Thy-1 and neural cell adhesion molecule (N-CAM) are up-regulated (Thompson *et al.,* 1984; Pleasure *et al.,* 1992) as is the polysialic acid associated with N-CAM and the sialytransferase enzyme that generates it (Kojima *et al.,* 1996). A surface antigen EX-1 is induced (Muller-Husmann *et al.,* 1994) and an epithelial membrane protein 1 which is a surface molecule contributing to myelin compaction is up-regulated (Wulf and Suter, 1999). Curiously, a high-mobility group (HMG) protein, normally a DNA binding protein, appears on the cell surface of neuroblastoma cells (Passalacqua *et al.,* 1998).

Neuronal induction also involves gap junctional communication because the inhibition of communication prevents the induction of neuronal markers MAP2 and neurofilament 200 (NF200) and the number of neurons is reduced (Bani-Yaghoub *et al.,* 1999). Presumably it is the early events of induction that were inhibited in these experiments because connexin 43 declines during RA-induced differentiation along with a reduction in dye coupling (Bani-Yaghoub *et al.,* 1997; Belliveau *et al.,* 1997).

The gene for the potassium channel, *IRK1,* is induced and the neuroblastoma cells used in these experiments become more hyperpolarized (Arcangeli *et al.,* 1998; Tonini *et al.,* 1999). The glutamate transporter appears (T. Matsuoka *et al.,* 1997; Dunlop *et al.,* 1998; Heck *et al.,* 1997b), cytosolic calcium oscillations are generated (Gao *et al.,* 1998a), and calcium channels are expressed (Gao *et al.,* 1998b). $GABA_A$ receptors, opioid receptors, glycine receptors, and nicotinic acetylcholine receptors are induced (Lin *et al.,* 1996; Cauley *et al.,* 1996; Reynolds *et al.,* 1996; T. Matsuoka *et al.,* 1997; Beczkowska *et al.,* 1997; Heck *et al.,* 1997a; Neelands *et al.,* 1998). Proteins associated with synapses such as synaptophysin, synaptobrevin, synaptotagmin, Rab3a, and synapsin appear (Finley *et al.,* 1996; Pleasure *et al.,* 1992; Strubing *et al.,* 1995; Berger *et al.,* 1997).

There have been several investigations of the changes induced in expression of the neurotrophin receptors and the expression of neurotrophins themselves, but there seems to be little uniformity in the results. The low-affinity nerve growth factor (NGF) receptor is consistently up-regulated (Haskell *et al.,* 1987; Scheibe

and Wagner, 1992; Ehrhard *et al.,* 1993; Cosgaya *et al.,* 1996; Takahashi *et al.,* 1999; Salvatore *et al.,* 1995), resulting in increased NGF binding at the cell surface, but TrkA, the high-affinity NGF receptor, has been shown to be either up-regulated (Haskell *et al.,* 1987; Lucarelli *et al.,* 1995; Hill and Robertson, 1997; Salvatore *et al.,* 1995) or not affected (Cosgaya *et al.,* 1996; Encinas *et al.,* 1999; Takahashi *et al.,* 1999). The mRNA for NGF itself is either up-regulated (Wion *et al.,* 1987) or not affected (Nakanishi *et al.,* 1996). TrkB has been almost uniformly shown to be induced (Kaplan *et al.,* 1993; Encinas *et al.,* 1999; Takahashi *et al.,* 1999; Lucarelli *et al.,* 1995; Hill and Robertson, 1997; Salvatore *et al.,* 1995; Renoncourt *et al.,* 1998) with one report of its decrease (Ehrhard *et al.,* 1993) and its neurotrophin, brain derived neurotrophic factor (BDNF), is not induced (Nakanishi *et al.,* 1996). TrkC is either induced (Encinas *et al.,* 1999; Renoncourt *et al.,* 1998) or not (Hill and Robertson, 1997) or prevented from disappearing (Takahashi *et al.,* 1999) and its neurotrophin, neurotrophin 3 (NT-3), is not induced (Nakanishi *et al.,* 1996). Finally, the ciliary neurotrophic factor (CNTF) has been shown to be induced (Nakanishi *et al.,* 1996); the cell surface receptor tyrosine kinase *ret,* which is the receptor for glial cell derived neurotrophic factor (GDNF) is up-regulated (Bunone *et al.,* 1995); and the tumor necrosis factor receptors are up-regulated (Chambout-Guerin *et al.,* 1995).

## 4. Extracellular Events

Some interesting experiments have demonstrated the importance of cell–cell signaling via secreted factors during RA induction of neuronal differentiation. Using GFP as a lineage label, it has been shown that coculturing an embryonal carcinoma cell line that produces only neurons with another line that produces neurons, astrocytes, and fibroblasts will induce the neuron-only cell line to produce astrocytes as well (Jostock *et al.,* 1998). This function is not found in conditioned medium so may be bound to an extracellular matrix molecule such as laminin. What could this factor(s) be? Some extracellular factors that have an effect on EC cells have been identified; for example, activin A is a secreted signaling molecule but it inhibits the RA induction in P19 and neuroblastoma cells (Hashimoto *et al.,* 1990; van den Eijnden-van Raaij *et al.,* 1991). Paradoxically, this activin inhibition occurs even though one of the activin receptors is induced by RA (Shoji *et al.,* 1998) and activin and RA act synergistically to induce the levels of *c-jun* (Momoi *et al.,* 1992) and the activity of voltage-dependent calcium channels (Fukuhara *et al.,* 1997). Nevertheless, this activin inhibition *in vitro* mimics the negative action of activin on the developing nervous system *in vivo*.

The mRNA for the cytokine interleukin 15 becomes alternatively spliced (Satoh *et al.,* 1998), transforming growth factor $\beta 1$ (TGF-$\beta 1$) mRNA is up-regulated (Cosgaya *et al.,* 1997), and a novel member of the TGF-$\beta$ family, called *lefty,* which may function as an intercellular signaling molecule is induced in P19 cells (Oulad-Abdelghani *et al.,* 1998). Several of the *Wnt* genes are induced in P19 cells,

embryonic stem cells, and human neuroblastoma cells (Smolich and Papkoff, 1994; Bain *et al.,* 1996; Wakeman *et al.,* 1998). The *Wnt*s are also secreted modulators of cell–cell interactions. When one *Wnt* in particular, *Wnt-1,* was overexpressed then several other *Wnt* genes were induced without RA and the loss of a particular cell surface antigen, SSEA-1, was also observed (Smolich and Papkoff, 1994). Despite the fact that all cells in the RA-treated culture dish would receive an RA signal, these observations mean that further intercellular signaling takes place via *Wnt-1,* leading to MAP2 induction and neural differentiation.

Thrombospondin is another extracellular matrix molecule that supports neurite outgrowth in tissue culture and it is induced by RA in neuroblastoma cells (Castle *et al.,* 1992). Treatment with an anti-thrombospondin antibody inhibited the RA-induced neurite outgrowth. Treatment with an anti-thrombospondin antibody and an anti-laminin antibody inhibited neurite outgrowth to an even greater degree.

Changes in the extracellular matrix are also bound to be important for neurite outgrowth. Thus, two low molecular weight, heparin-binding proteins have been described that are induced by RA and stimulate neurite outgrowth. One is called RI-HB, for retinoic acid–induced heparin binding protein (Raulais *et al.,* 1991), and the other is called MK, for midkine (Muramatsu and Muramatsu, 1991; Muramatsu, 1993; Nurcome *et al.,* 1992). MK is certainly found on the surfaces of neural cells and thus could fulfill a natural function, but it is also widely expressed in other regions of the embryo and is thus not unique to neurite producing cells (Kadomatsu *et al.,* 1990; Muramatsu *et al.,* 1993). The same is true for RIHB, which is found in the very early embryo throughout the blastodisc and in all of the germ layers following gastrulation; following neurulation its expression *decreases* in the neural tube before it does in other tissues (Cockshut *et al.,* 1994). However, glycosaminoglycans such as heparin enhance the effects of RA on the number of cells forming neurites (Lesma *et al.,* 1996). RA also induces an increase in the $\alpha^1/\beta^1$ integrin receptor, but does not change the $\alpha^3/\beta^1$ integrin receptor (Rossino *et al.,* 1991). Since the $\alpha^1/\beta^1$ integrin receptor is the major laminin receptor and these RA-treated cells subsequently demonstrate an increased neurite response to laminin [in contrast to NGF, insulin, or 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-treated cells] these results suggest a mechanism of action via extracellular matrix molecules. Indeed, laminin itself is up-regulated by RA in EC cells and neuroblastoma cells (Wang *et al.,* 1985; Castle *et al.,* 1992). In addition, tenascin is also up-regulated in neuroblastoma cells along with the $\alpha^1$, $\beta^1$, and $\alpha^v$ integrin subunits (Linnala *et al.,* 1997). Because these integrins could also act as tenascin receptors this is a parallel mechanism of action. Other extracellular matrix molecules induced include matrix metalloproteases (Chambaut-Guerin *et al.,* 2000) and metallothionein-3 (Faraonio *et al.,* 2000).

After this host of molecular changes, the cells (or a proportion of them—the ones that do not become astrocytes, oligodendrocytes, fibroblast-like cells, or apoptose) become true neurons and undergo electrophysiological maturation. Glutamate receptor channels are induced, there is a change in the $GABA_A$ receptor subtypes

similar to those found in the mature CNS, synaptic proteins such as synaptophysin and synapsin appear (see Section II.A.3), and synapses can be seen in the electron microscope, the neurotransmitters for both inhibitory and excitatory synaptic transmission are produced and the neurons generate action potentials and establish electrical connectivity (Bain *et al.,* 1995; Finley *et al.,* 1996; MacPherson *et al.,* 1997; Staines *et al.,* 1994; Turetsky *et al.,* 1993; Younkin *et al.,* 1993; Neelands *et al.,* 1999; Dunlop *et al.,* 1998).

### 5. Type of Neurons Produced

Characterization of these neurons with regard to neurotransmitter phenotype has produced differing results. As mentioned above, some report the induction of a catecholamine phenotype in P19 cells (Sharma and Notter, 1988) while others have found that a cholinergic phenotype is induced in P19 cells (McBurney *et al.,* 1988; Parnas and Linial, 1995; Coleman and Taylor, 1996), PC12 cells (Matsuoka *et al.,* 1989), and human neuroblastoma cells (Casper and Davies, 1989; Sidell *et al.,* 1984; Hill and Robertson, 1997). Other neurotransmitter phenotypes induced include the dopaminergic phenotype (Farooqui, 1994), the GABAergic phenotype (Fraichard *et al.,* 1995; Bain *et al.,* 1996; Lin *et al.,* 1996; Neelands *et al.,* 1998; T. Matsuoka *et al.,* 1997), the glutaminergic phenotype (MacPherson *et al.,* 1997; T. Matsuoka *et al.,* 1997; Heck *et al.,* 1997b; Dunlop *et al.,* 1998; Turetsky *et al.,* 1993; Younkin *et al.,* 1993), the glycinergic phenotype (Strubing *et al.,* 1995; Heck *et al.,* 1997a), or the opioid phenotype (Beczkowska *et al.,* 1997; Tryoen-Toth *et al.,* 2000). A detailed study of these characteristics in one cell type, P19 cells, has revealed that the induced neuronal cells, as might be expected from the above distribution, are highly diverse with several neurotransmitter phenotypes induced in the same population (GABA, neuropeptide Y, serotonin, and others) (Staines *et al.,* 1994). Similar results were obtained with ES cells (Renoncourt *et al.,* 1998). So it seems that no one single neurotransmitter phenotype is induced by RA, but instead many are induced.

However, most studies have concluded that the neurons show the characteristics of CNS neurons rather than peripheral nervous system (PNS) neurons (Pleasure *et al.,* 1992; Dunlop *et al.,* 1998; Strubing *et al.,* 1995; Pahlman *et al.,* 1984). One detailed investigation using a panel of antibodies specific to neuronal subtypes which express different combinations of homoebox proteins within the spinal cord has shown that RA induces the expression of Pax-6 (characteristic of ventral CNS), sonic hedgehog (ventral floorplate), Islet (motoneurons), HB9 (motoneurons), Lim3 (motoneurons), Phox2b (motoneurons), Lim1/2 (ventral interneurons), and En-1 (ventral interneurons) (Renoncourt *et al.,* 1998). So RA seems to induce CNS neurons of ventral characteristics.

Finally, most studies have concluded that the neurites that are produced are more likely to be dendrites than axons (Kozireski-Chuback *et al.,* 1999; Wu *et al.,* 1998), which is surprising since a neuron would be expected to have both (although only

one axon, of course). A careful cell biological study using antibodies to proteins found only in dendrites such as MAP2 or to proteins found only in axons such as synaptophysin and following the movement of the cell nucleus has concluded that the neurons are perfectly normal with one axon and several dendrites (Berger *et al.,* 1997).

Thus the induced neuron is a ventral CNS neuron with any one of many neurotransmitter phenotypes.

### 6. Necessary Gene Products Involved in RA-Induced Differentiation

In conclusion, we can bring together those inductive or repressive events that have been shown to be necessary and sufficient for RA-induced neurite outgrowth. They are grouped together in Table I.

## B. Dissociated or Explanted Neuronal Cells

Studies on the mechanism of action of RA on neurons themselves have been less extensive but have revealed similar effects on the cellular machinery to those described above for embryonal carcinoma and neuroblastoma cells.

Concerning neurotransmitters, RA can change the phenotype. In cultured newborn rat sympathetic neurons or rat septal cells, it is a consistent finding that RA increases choline acetyltransferase activity and the level of acetylcholine (Bussiere *et al.,* 1995; Szujowicz *et al.,* 1999) while reducing the activity of the catecholamine synthetic enzymes tyrosine hydroxylase and dopamine beta hydroxylase (Berrard *et al.,* 1993; Kobayashi *et al.,* 1994a). A norepinephrine transporter is also induced (I. Matsuoka *et al.,* 1997).

TABLE I
Required Steps in RA-Induced Neuronal Differentiation

| Receptors | Transcription factors | Intracellular signals | Cytoplasmic proteins | Extracellular molecules/structures |
|---|---|---|---|---|
| | Brn-2 | PKC$\varepsilon$ | | |
| RAR$\alpha$/RXR | NF-kB | PTP$\alpha$ | MAP2 | Thrombospondin |
| RA | Stra13 | Ceramide | Presenilin | Laminin |
| RAR$\gamma$/RXR | DAN | $^{\text{decreased}}$SHP-1 | C term APP | Gap junctions |
| | SOX1 | | | |

*Note:* The requirement for these molecules is defined by the observations that in their absence RA-induced differentiation does not take place, or when overexpressed the requirement for RA is overcome.

With regard to neurotrophins and receptor expression, in embryonic chick sympathetic neurons, for example, RA induces the high-affinity NGF receptor, TrkA, which then permits the neurons to become NGF responsive (Rodriguez-Tebar and Rohrer, 1991; van Holst *et al.,* 1997). There is no effect on TrkC, the low-affinity NGF receptor, or TrkB (van Holst *et al.,* 1995). These cells express RAB$\beta$, RAR$\gamma$, and RXR$\gamma$ mRNAs and, in particular, increasing levels of RAB$\beta$ correlate with the development of NGF responsiveness (Plum and Clagett-Dame, 1996). However, van Holst *et al.* (1997) have concluded that this effect on TrkA is mediated by RAR$\alpha$ because a RAR$\alpha$-selective agonist mimics the effect of RA and it is blocked by a RAR$\alpha$ antagonist. The survival of embryonic chick ciliary ganglion neurons is enhanced because RA interacts with a heparin-binding growth factor (Hendry and Belford, 1991), a similar molecule to the MK and RI-HB proteins described above, but this effect may also be mediated by an induction of the CNTF receptor (Wang and Halvorsen, 1998). In chick vestibular ganglion explants RA induces *ret,* the neurturin receptor, and thus allows neurites to be induced (Hashino *et al.,* 1999) and in retinal ganglion cells TrkB is induced, thereby allowing BDNF to induce axonal regeneration (Mey and Rombach, 1999).

This effect of RA seems not to occur in cultured trigeminal sensory neurons of the mouse embryo, however (Paul and Davies, 1995). Indeed, opposite effects have been reported using mouse embryonic sympathetic neurons. TrkA was down-regulated and TrkC was up-regulated (Wyatt *et al.,* 1999), but the effects of RA were mimicked by a RAR$\alpha$ agonist. This seems to be a consistent finding with these primary neuronal cultures.

In newborn rat sympathetic neurons RA seems again to do different things to the neurotrophin receptors from that seen in chicks as it induces change in their neurotrophin responsiveness from NGF to BDNF (Kobayashi *et al.,* 1994b). To facilitate this, TrkB is induced and TrkA is down-regulated. But TrkC is also induced and the cells become NT-3 responsive as well (Kobayashi *et al.,* 1998). Conforming with the chick, RA up-regulates *ret* in rat sympathetic neurons, and this effect is mimicked by an RAR$\alpha$ agonist (Thang *et al.,* 2000). During this induction of *ret,* RAR$\alpha$ and RAR$\beta$ are up-regulated.

Other molecular effects on primary cultures of neurons mimic those seen in EC cells described above. Cell death is induced in a population of mouse embryonic cerebral cortex cells (Okazawa *et al.,* 1996), and in rat embryonic hippocampal cells the APP protein is up-regulated (Yang *et al.,* 1998; Hung *et al.,* 1992). *Brn-3b* is up-regulated by RA in mouse embryonic trigeminal neurons (Wyatt *et al.,* 1998) and adenylate kinase is up-regulated in rat cerebral cortex neurons (Inouye *et al.,* 1998). Dopamine D2 receptors are up-regulated in cultures of rat striatal neurons (Valdenaire *et al.,* 1998) and the response is mediated by a RARE. RA induces tissue transglutaminase in adult rat sympathetic cells (Ando *et al.,* 1996), this molecule being involved in the stabilization of newly formed synapses.

Glial cells and oligodendrocytes have also been used for RA studies with somewhat contrasting results. Some report the down-regulation of myelin basic protein, suggesting an inhibitory effect on maturation of myelin-producing oligodendrocytes (Tryoen-Toth *et al.,* 2000), whereas others have reported a stimulation (Pombo *et al.,* 1999). In support of the latter result, RA seems to play a role in the normal differentiation of oligodendrocytes as it slows down cell division and induces the differentiation of oligodendrocyte precursor cells (Barres *et al.,* 1994). Not surprisingly this action requires the activation of gene transcription because the use of synthetic retinoids that do not transactivate on the RARE does not differentiate the oligodendrocytes (Tokumoto *et al.,* 1999). Finally, with regard to molecules involved in Alzheimer's disease which have been studied in EC cells, one study has used astrocytes and found that the ApoE protein is up-regulated by RA (Garcia *et al.,* 1996).

Neural crest cells are another neural cell type that has been used for studies of the effects of RA. In tissue culture, neural crest cells differentiate into a wide variety of cell types which reflects their derivatives in the embryo (Baroffio *et al.,* 1988, 1991) and it has long been known that RA inhibits the migration of cultured crest *in vitro* (Thorogood *et al.,* 1982; Smith-Thomas *et al.,* 1987; Webster *et al.,* 1986). However, 1- to 10-n*M* retinoic acid stimulates the differentiation of neurons in such cultures by selectively promoting neuronal precursor proliferation and survival (Henion and Weston, 1994). Furthermore, RA stimulates one particular type of neuron, the adrenergic neuron, to differentiate (Dupin and Le Douarin, 1995; Rockwood and Maxwell, 1996b) and this effect seems specific to the RAR$\alpha$ pathway because an RAR$\alpha$-selective compound also induces this effect, whereas 9-*cis*-RA does not (Rockwood and Maxwell, 1996a).

The major reason for using primary cultures of neurons or explanted neural tissue for RA studies has been not to see whether the molecular effects are the same as in EC cells, but to see whether RA could induce more or longer neurites. The implication of such results would be that perhaps RA does this in the embryo endogenously during development or that RA could be used to stimulate regeneration of neurites postnatally. Thus, chick embryo dorsal root ganglion (DRG) explanted into normal medium exhibited no neurite extension, but when treated with RA, neurites were produced (Haskell *et al.,* 1987). This same result has been seen with amphibian spinal cord explants (Hunter *et al.,* 1991), chick embryo sympathetic neurons (Avantaggiato *et al.,* 1996), fetal mouse spinal cord, human spinal cord (Quinn and De Boni, 1991), embryonic rat cerebellum (Yamamoto *et al.,* 1996), and embryonic mouse cerebral cortex (Ved and Pieringer, 1993). Using mouse neonatal DRG, RA stimulated not more, but longer neurites (Quinn and De Boni, 1991). Experiments on dissociated embryonic chick spinal cord revealed that RA could accomplish both effects, namely, increase the number and the length of neurites (Maden *et al.,* 1998a). In dissociated embryonic rat spinal cord cultures, RA promoted the survival of the neurons (in contrast to inducing apoptosis of EC cells described above) and increased neuritic density (Wuarin *et al.,* 1990). In

particular, RA increased the survival of cholinergic neurons and had no effect on GABAergic neurons, whereas the effect on neurite outgrowth was seen on most neurons regardless of their neurotransmitter phenotype (Wuarin and Sidell, 1991).

The mechanism of action of RA in promoting neurite outgrowth has been investigated in embryonic and adult mouse dorsal root ganglia. RA promotes neurite outgrowth in both of these cell types and RAR$\beta$ is up-regulated (Corcoran *et al.,* 2000). In contrast to the result described above with a RAR$\alpha$ agonist, in this work a RAR$\beta$ agonist mimicked the effects of RA. Furthermore when NGF was added to the cultured ganglia, RAR$\beta$ was also up-regulated as well as an enzyme that makes RA, namely, retinaldehyde dehydrogenase type 2 (RALDH2) (Corcoran and Maden, 1999). This, along with the data described above, suggests that there is a close relationship between RA and NGF in their mechanism of action on neurons.

The evidence above suggests that RA is an important regulator of neurite outgrowth in development. However, the results might not reflect the natural situation, but be an artifact of an artificial inducer of differentiation. The following data answer this doubt, first by showing that RA and its molecular machinery (RARs, RXR, binding proteins) are present in the developing nervous system and, secondly, that when either the supply of RA is stopped or its molecular machinery is disrupted, then nervous system development is disrupted.

## III. The Presence of RA and Its Molecular Transducers in the Developing CNS

### A. Detecting RA Itself

The most direct method for determining the presence or absence of retinoids in the nervous system is to use high-pressure liquid chromatography (HPLC). Individual retinoids can be identified according to their elution time and UV spectrum. RA first appears in the mouse embryo at the mid-primitive streak to late allantoic bud stages (day 7.5) (Ulven *et al.,* 2000) and is present in the stage 5–8 bird embryo (Dong and Zile, 1995). However, only three measurements of endogenous retinoids in the CNS have been made by this method because of the difficulty of obtaining enough tissue from which to extract the retinoids. The larval amphibian spinal cord has been shown to contain all-*trans*-RA, all-*trans*-retinol, and 13-*cis*-RA (Hunter *et al.,* 1991). In mammals, the mouse embryonic day 10.5 and day 13 CNS shows an interesting differential distribution of retinoids. The forebrain, midbrain, and hindbrain contain extremely low levels of all-*trans*-RA, but the neural tube that will develop into the spinal cord contains the highest level in the whole embryo (Horton and Maden, 1995). We have also recently confirmed that the developing spinal cord in the chick embryo can synthesize RA from retinol (Maden, unpublished

data, 2000). Therefore, somewhere in the posterior part of the hindbrain or at the hindbrain/spinal cord junction there is a sudden step from very low levels to very high levels of RA. This has been confirmed by two other methods, transgenic embryos and reporter cells (see Sections III.B and III.C).

The developing eye of the mouse embryo also contains retinoids. Retinas from day 13–14 and day 15–17 mouse embryos were used to detect all-*trans*-RA and 13-*cis*-RA (McCaffery *et al.,* 1993), there being surprisingly high levels of 13-*cis*-RA. The retinoic acid concentration of embryonic retinas decreased 4-fold in the adult. In a subsequent study (Mey *et al.,* 1997), no 13-*cis*-RA was detected and it was shown that at early stages (day 4 and day 5) there were higher levels of RA in the ventral retina compared to the dorsal retina, but that this situation reversed at later stages. Indeed, cytosolic fractions of dorsal and ventral retinas from day 14–15 embryos were examined for retinoic acid synthesis and it was found that the dorsal part gave a 10-fold higher peak than the ventral part (McCaffery *et al.,* 1992). However, this is in contrast to the F9 reporter cell data described below: ventral halves gave higher responses than dorsal halves. This contradiction was interpreted as a dorsoventral difference in the ability of the enzymes to metabolize at high retinaldehyde levels and that the ventral pathway became substrate inhibited in the HPLC studies.

The ability to detect the synthesis of RA using a "zymography assay" has led to the identification of several areas of the nervous system that can perform this activity in addition to the eye (McCaffery *et al.,* 1992, 1999; Mey *et al.,* 1997). These include the choroid plexus, the meninges of the spinal cord, the anterior pituitary, the olfactory bulb, and the corpus striatum (McCaffery and Drager, 1995; Yamamoto *et al.,* 1996). Interestingly, when the embryonic cerebellum is co-cultured with the choroid plexus, neurite outgrowth is stimulated (Yamamoto *et al.,* 1996), a stimulation that is mimicked by RA. These authors suggest that this might be the normal function of the choroid plexus during cerebellar development.

## B. Transgenic Embryos

Using a reporter construct containing the RARE from the RAR$\beta$ gene linked either to a minimal promoter or the RAR$\beta$ promoter itself and the $\beta$-galactosidase gene, transgenic zebrafish and mouse embryos have been created to indicate when and where RA is present in the embryo. In the zebrafish, even though the construct was injected into the 1 and 2 cell embryo, the first localization was not observed until the surprisingly late stage of 18–21 somites (Marsh-Armstrong *et al.,* 1995) Then the RA-induced $\beta$-galactosidase expression was concentrated in the anterior trunk region in tissues that included the spinal cord and floor plate. The lack of early expression must reflect the limited sensitivity of that particular transgene in the zebrafish embryo because another reporter construct using Gal4 is activated at the end of gastrulation in a posterior dorsal domain (Joore *et al.,* 1997).

Several groups have generated transgenic mice with these varying RAR$\beta$ promoter constructs (Mendelsohn *et al.,* 1991; Reynolds *et al.,* 1991; Rossant *et al.,* 1991; Shen *et al.,* 1992; Balkan *et al.,* 1992). In these embryos, activation of the reporter construct began at the early somite stage, when the neural tube had just commenced closure and there was a sharp anterior boundary of expression at the level of the first somite, at the joint between the hindbrain and the spinal cord and expression ceased at about the level of the posterior neuropore (although it did not extend so far posteriorly in the studies of Shen *et al.* and Mendelsohn *et al.*) (Fig. 1A). This is entirely consistent with the distribution seen in endogenous RA measurements. In the head region only the optic eminences and the olfactory mesenchyme showed any transgene activity and, later, the eye itself (both lens

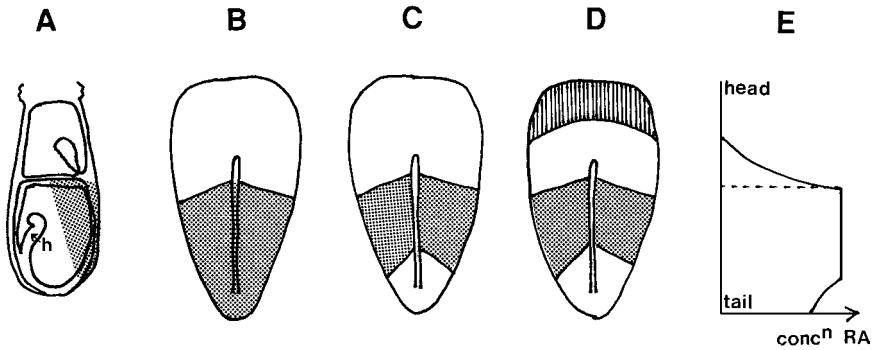



FIG. 1 Endogenous RA and enzymes in the early embryo. (A) RA in the day 7.5 mouse embryo as assessed in the RARE*lacZ* transgenic mouse of Rossant *et al.* (1991). The anterior of the embryo is to the left (h = headfold) and the posterior is to the right. The expression of the reporter gene is shown in stippling and is in the posterior part of the embryo. (B) Detection of RA (stippled area) in the stage 4/5 chick embryo assessed by placing pieces of embryo on an F9 reporter cell line as described in Maden *et al.* (1998). Anterior is at the top and posterior is at the bottom; the primitive streak is in the midline. The stippled area represents parts of the embryo where RA can be detected and there is a sharp on–off border just posterior to the node that will eventually be located at the level of the first somite. (C) Expression of RALDH2 (stippled area) in the stage 4/5 chick embryo. Both mRNA (Blentic and Maden, unpublished, 2000; Swindell *et al.,* 1999) and protein (Maden and McCaffery, unpublished, 2000) have the same distribution and the sharp on–off border of RALDH2 is the same as the border of RA in part (B). (D) Expression of RALDH2 (stippled) and CYP26 (striped) in the stage 4/5 chick embryo (Swindell *et al.,* 1999; Blentic and Maden, unpublished, 2000). CYP26 is expressed in the presumptive forebrain and midbrain, the gap between the two expression domains is the presumptive hindbrain and as in part (C) the RALDH2 expression is in the posterior of the embryo with an anterior border at the level of the first somite, encompassing the presumptive spinal cord. (E) Graphical representation of the concentration of RA along the head-to-tail axis of the early embryo. The horizontal axis represents the concentration of RA and the vertical axis represents the embryonic axis. At the anterior (head end) of the embryo there is no RA due to the presence of CYP26. Across the presumptive hindbrain there is a gradient that is generated by RA released from the level of the first somite. In the trunk of the embryo the levels of RA are high, and then at the tail end the level probably declines because RALDH2 is not expressed in this location.

and retina) was positive. This confirms that the forebrain (except for the eyes), midbrain, and most of the hindbrain develop in the absence of RA with a strong presence of RA in the spinal cord.

Within the cross section of the spinal cord the data generally show that the transgene activity was initially present strongly throughout the dorsoventral axis, but was lost from the central region of the cord, thereby leaving expression in the dorsal one-third, the mantle layer, and the motoneurons and their axons. Another study using a different reporter transgene (Gal4) linked to either a RAR or RXR has found that both reporters were first expressed in the ventral part of the spinal cord and then the activity spread dorsally and then disappeared (Solomin *et al.,* 1998). This pattern occurred first with the RAR reporter followed half a day later by the same pattern with the RXR reporter.

With regard to other parts of the nervous system, some of these studies found that the dorsal root ganglia were positive for the RAR transgene (Mendelsohn *et al.,* 1991), but others found no expression (Rossant *et al.,* 1991) and some suggested that only the surrounding meningeal layer is positive (Reynolds *et al.,* 1991). All of these studies agree, however, that the activity of the transgenes, both RAR and RXR, within the spinal cord is expressed throughout the anteroposterior extent of the spinal cord at day 10.5 (but not in the forebrain, midbrain, or hindbrain), and becomes restricted to two distinct anterior and posterior domains within the spinal cord at day 12.5 of development (Colbert *et al.,* 1993; Solomin *et al.,* 1998). These domains correspond to the brachial and lumbar enlargements where the limb buds will develop, that is C1–T2 and T13–S2 with a negative region between (Colbert *et al.,* 1995; Rubin and La Mantia, 1999) and they are the "hot spots" of retinoic acid synthesis (see below) (McCaffery and Drager, 1994b). The association of high RA levels with the cervical and lumbar enlargements is confirmed by the observation that these regions are where one of the enzymes that generate RA, namely, RALDH2, is specifically localized within the spinal cord (see Section III.D.1). This is an intriguing observation because these are the areas where the limbs will grow out and there are more and different classes of motoneurons here compared to elsewhere in the cord. It suggests that RA may contribute to the enhanced neuronal survival found here or may be responsible for the regulation of differentiation of particular classes of motoneuron types (see Section III.D.1).

The region of retinoid activity at the rostral end of the embryo is concerned with the development of the olfactory region and the eye. There is a very localized area of activity in the ventrolateral neuroepithelium of the forebrain vesicle adjacent to the olfactory placode which later becomes localized to the olfactory epithelium and the olfactory nerve itself (LaMantia *et al.,* 1993). The cranial mesenchyme between the olfactory epithelium and the ventrolateral forebrain activates F9 reporter cells (see Section III.C) even though it does not light up in the transgenic reporter mouse. Nevertheless this whole area seems to be a site of retinoid activity.

So too is the developing eye, which as described above synthesizes RA and has a remarkable distribution of RA synthesizing and metabolizing enzymes (see Section III.D.1). The neuroepithelium of the optic vesicle, surrounding mesenchyme, and the epithelial lens placode are all activated in the reporter mouse from day 8.75 onward (Enwright and Grainger, 2000). Later in development this uniform blueness is broken up by the disappearance of transgenic activity in the lens and the appearance of an RA-poor stripe in the eye that spans the nasotemporal axis at the equator (Wagner *et al.,* 2000). The retinal genglion axons which leave the dorsal and ventral hemispheres of the eye to connect to the tectum are also blue and the can be traced all the way to their specific topographical locations. These results imply that the retinal ganglion axons contain RA as well as their cell bodies and perhaps use RA as a signaling system for topographical navigation.

## C. Reporter Cells

Reporter cells have been generated by transfecting F9 cells (Wagner *et al.,* 1992; Sonneveld *et al.,* 1998) or L-$C_2A_5$ cells (Colbert *et al.,* 1993) with these RARE-$\beta$-galactosidase constructs. Such cells respond to the presence of RA in the medium or to the release of RA from a piece of embryonic tissue that has been placed on the cells by turning blue after histochemical staining for *LacZ* exactly as whole embryos described above do. Using this technique in the zebrafish embryo, a similar picture emerged to the transgenic studies (Marsh-Armstrong *et al.,* 1995). There was a gradient of RA along the trunk (whole trunk sections were used rather than the CNS itself because of the small size) with the highest levels in the anterior regions (at the level of somites 7–9) and the hindbrain region showed only very low levels.

Studies of the chick embryo (Maden *et al.,* 1998b) have produced the same picture as the transgenic mice described above. After neural tube formation no activation of F9 cells occurs when forebrain, midbrain, or hindbrain neuroepithelium is placed onto the cells, but high activation is found from the spinal part of the neural tube, thus there is a sharp on–off border of activity (Fig. 1B). No difference could be detected along the length of the spinal cord at these early stages, but later the cervical and lumbar enlargements show higher activity than the thoracic region in between.

In the mouse embryo, a similar picture emerged when whole embryos were placed onto these F9 reporter cells (Ang *et al.,* 1996). RA could first be detected at late streak stages (day 7.5) arising from the embryonic tissue, not the extraembryonic tissue. One day later RA was detected adjacent to the trunk region with an anterior border in the hindbrain/spinal cord region and a posterior border toward the posterior end but not reaching it. The highest region of activity was located in the prospective forelimb bud region. When the head was dissected into its component

parts, the anterior hindbrain region and the posterior midbrain-anterior forebrain region were inactive, but there was retinoid activity in the craniofacial region where the optic eminences arise. A comparison of several day 11 mouse embryonic tissues has shown that the cerebral vesicles and mesencephalon have very low levels of activity whereas the spinal cord has very high levels (McCaffery *et al.,* 1992). The absence, or very low levels, of detectable retinoids in the forebrain has also been observed by Wagner *et al.* (1992) in rat embryos and LaMantia *et al.* (1993) in mouse embryos. However, there are localized areas of activity underlying this general negativity of the forebrain such as the mesenchyme, which lies between the ectodermal olfactory placode and the olfactory bulb of the forebrain (LaMantia *et al.,* 1993) and the eye, as noted above in the transgenic mice. Within the developing eye both the optic vesicle and the epithelial lens placode generate RA (Enwright and Grainger, 2000). Subsequently, the ventral retina generates a higher level of activity than the dorsal retina (McCaffery *et al.,* 1992, 1999).

The uniform levels of retinoids detected in the early mouse spinal cord changes later on in development such that there is a concentration of retinoid activity in the brachial and lumbar eminences of the CNS as assayed by the F9 reporter cells, exactly as found in the transgenic mice (Colbert *et al.,* 1993; McCaffery and Drager, 1994b). These are the "hot spots" of retinoic acid synthesis in the spinal cord.

In the cross section of the spinal cord Wagner *et al.* (1992) showed that the ventral floor plate activated the F9 reporter cells, but so too did the dorsal spinal cord in rat embryos. This accords with experiments on the synthesis of RA by the chick embryo spinal cord where it was shown that the rate of synthesis by the ventral floor plate was not very different from that of the dorsal spinal cord (Wagner *et al.,* 1990). However, studies in the mouse embryo have shown that at levels intermediate between the brachial and lumbar eminences there was an equivalent synthesis of RA in dorsal and ventral halves, but at the "hot spots" themselves there was a vast excess of RA generated in the ventral halves (McCaffery and Drager, 1994b). This is not the result that was obtained in the transgenic animals nor in the synthesis experiments in the chick embryo; in fact, quite the opposite, and to reconcile the data these authors have suggested that the levels of RA are so high in the ventral half of the cord at the eminences that the reporter is turned off.

A final region of the CNS that has been shown to generate RA using reporter cells is the lateral ganglionic eminence (LGE) of day 12.5 mouse embryos, suggesting a role for RA in the development of the striatum (Toresson *et al.,* 1999). In contrast, the medial ganglionic eminence (MGE) was negative. Furthermore, the cell type that seems to be responsible for this activity was not the neurons, but the glia. In day 15 mouse embryos the same difference between the negative MGE and the strongly positive LGE is present and the brain meninges and the retina are also positive (Li *et al.,* 2000). In contrast, the hippocampus, cerebellum, and superior colliculus were negative.

## D. RA-Synthesising Enzymes

### 1. Retinaldehyde Dehydrogenases

By studying the enzymes that generate RA from retinol, the medium-chain alcohol dehydrogenases (ADHs) and the RALDHs, we can gain some important insights into whether RA acts on embryonic cells in an autocrine fashion or a paracrine fashion. If RA is generated by the cells that require it for their own use, then the distribution of the RALDHs should be the same as the distribution of RA described above, but it could be that the RA in the CNS is provided by an adjacent tissue, in which case the enzymes would have a different distribution.

In the eye, the former seems to be the case. As we saw above, the transgenic reporter mouse gave a picture of a "blue" eye with a white stripe across it. This distribution is generated by at least four enzymes. In the dorsal half of the eye there is an aldehyde dehydrogenase known as RALDH1 (originally known as AHD2) (Haselbeck *et al.,* 1999; McCaffery *et al.,* 1999), which was initially identified in studies characterizing the different proteins occurring in dorsal and ventral halves of the eye rather than in studies of RA metabolism. A protein found in the dorsal retina turned out to be an aldehyde dehydrogenase, then called AHD-2 (McCaffery *et al.,* 1991). The same enzyme is also exclusively located in the dorsal part of the developing eye of the chick embryo (Godbout *et al.,* 1996). Surprisingly, on the F9 reporter cells the ventral retina of the mouse embryo generated more RA than the dorsal retina (McCaffery *et al.,* 1992, 1999), suggesting the presence of an additional enzyme in the ventral part. This ventral enzyme is RALDH3 (formerly called V1 and also known as ALDH6) (Li *et al.,* 2000; Mic *et al.,* 2000). The RA-low stripe in the middle is generated by an enzyme that metabolizes all-*trans*-RA into 4-*oxo*-RA, 4-OH-RA, 18-OH-RA, and 5,8 *epoxy*-RA (White *et al.,* 1996, 1997; Fujii *et al.,* 1997; Abu-Abed *et al.,* 1998; Swindell *et al.,* 1999), namely, CYP26 (McCaffery *et al.,* 1999; Fujii *et al.,* 1997). Finally another enzyme RALDH2 is present transiently at the ventral edge of the eye field of the mouse embryo (Niederreither *et al.,* 1997; McCaffery *et al.,* 1999; Haselbeck *et al.,* 1999; Li *et al.,* 2000; Mic *et al.,* 2000) and throughout the later retinal pigment epithelium in the chick embryo (Berggren *et al.,* 1999).

In the neural tube it may be that the second mechanism operates, the paracrine mode, because within the neural tube no enzyme has been described that mimics the distribution of endogenous RA with a sharp on–off border at the spinal cord–hindbrain junction. In fact, at early stages no enzymes have been found at all in the neural tube. The distribution of RALDH2, however, does show this striking border of expression at the correct level. It begins to be expressed at the same time that RA can first be detected in the embryo (Ulven *et al.,* 2000; Swindell *et al.,* 1999; Niederreither *et al.,* 1997; Maden and McCaffery, unpublished, 2000) and with a very similar distribution to that of endogenous RA (compare Figs. 1B and 1C), but it is expressed in the mesenchyme, not the neural tube. In the chick embryo

at the same time that RALDH2 first appears at stage 4 in the posterior part of the embryo, the enzyme that breaks down RA, CYP26, appears at the rostral end of the embryo in the presumptive forebrain and midbrain region (Swindell *et al.,* 1999). In between these two domains is the presumptive hindbrain (Fig. 1D). A similar expression of CYP26 has been seen in the mouse (Fujii *et al.,* 1997; de Roos *et al.,* 1999), but in the *Xenopus* embryo there is an extensive domain of CYP26 in the posterior of the embryo as well (de Roos *et al.,* 1999; Hollemann *et al.,* 1998). It therefore becomes an attractive idea, at least in the chick and mouse embryo, that RA could be generated from the level of the developing spinal cord, diffuses rostrally through the hindbrain, and is metabolized by CYP26 in the midbrain and forebrain. This situation would thereby generate a gradient of RA across the presumptive hindbrain which would be used to pattern the hindbrain (Maden, 1999a) (Fig. 1E). This would explain why the hindbrain is so peculiarly sensitive to perturbation after either excess RA, a deficiency of RA, or an alteration of the receptor expression levels (see Sections III.G and IV).

However, examining the tissues that express these two enzymes revealed that CYP26 was expressed in the neuroepithelium, but the RALDH2 was expressed in the mesenchyme and *not* the neuroepithelium of the developing spinal cord (Niederreither *et al.,* 1997; Swindell *et al.,* 1999; Berggren *et al.,* 1999; Haselbeck *et al.,* 1999). Therefore in order for the hindbrain RA gradient to be operative, RA would have to diffuse first into the neural tube from the adjacent mesenchyme where it is synthesized and then diffuse rostrally toward the site of CYP26 expression in the plane of the neuroepithelium (Fig. 2A). This seems to be placing a rather excessive demand on the directionality of diffusion gradients and it would be much more logical if RA were generated in the neuroepithelium of the spinal cord itself. Evidence to support the idea that there is a neural RALDH comes from two sources. Firstly, we have shown that the isolated chick embryonic neural tube can synthesize RA from retinol (Maden, unpublished, 2000). Secondly, retinol (which must have been converted within the tissue to RA) can induce certain subsets of interneurons characterized by the expression of the homeobox genes *Dbx1, Dbx2, Evx1/2* and *En* in explants of isolated, naïve neural plate long before the onset of RALDH2 expression (Pierani *et al.,* 1999). However, as yet there is no candidate for such an enzyme with an expression domain in the spinal cord and a sharp on–off border at the level of the hindbrain.

On the other hand, there is much evidence to show that the somites themselves, presumably via the RA that their RALDH2 synthesises, can affect patterning in the adjacent neural tube. For example, when brachial somites are transplanted caudally and placed next to thoracic spinal cord, the type of motoneurons found are changed from a thoracic type to a brachial type (Ensini *et al.,* 1998). Conversely, when cervical somites are grafted rostrally adjacent to the hindbrain the expression patterns of *Hoxb-4, Hoxa-3, cek-8,* and *Kreisler* are altered (Itasaki *et al.,* 1996; Grapin-Botton *et al.,* 1997; 1998) Evidence that RA is involved is that these gene inductive effects within the hindbrain are mimicked by the implantation

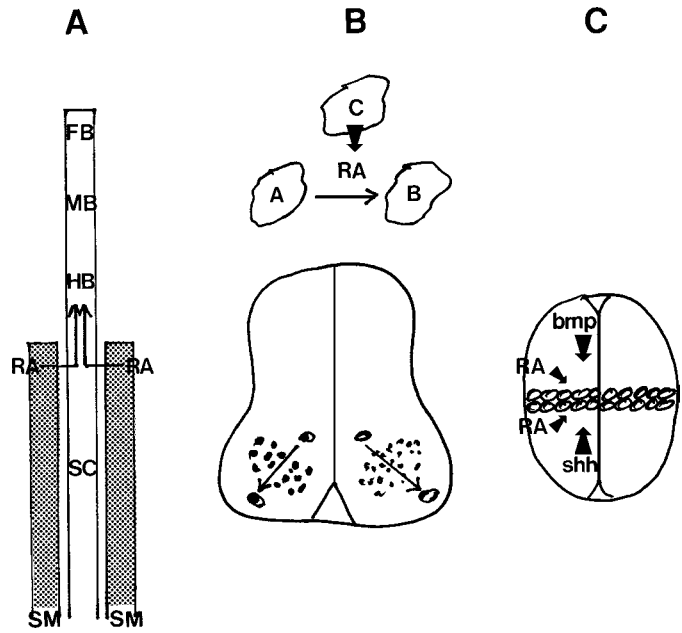



FIG. 2 Neuronal systems that require RA for their development. (A) The RA gradient shown in Fig. 1D is generated in a complex fashion. RALDH2 is expressed in the somitic mesoderm (SM) either side (or below at earlier stages) of the neural tube. RA is metabolized by CYP26 in the neuroepithelium of the presumptive forebrain (FB) and midbrain (MB). Therefore, in order for a gradient of RA to be generated across the presumptive hindbrain it must needs diffuse into the presumptive spinal cord (SC) from the adjacent mesoderm and then turn rostrally and diffuse across the presumptive hindbrain in the direction shown by the arrows. (B) RA functions later in the spinal cord to induce a subset of motoneurons known as $LMC_L$. Here a cross section is shown of the spinal cord, and RALDH2 is expressed by the early born motoneurons shown in the ventral part of the spinal cord as dots and at greater magnification as the cell marked C above. These cells produce RA, but they do not themselves become $LMC_L$s. The $LMC_L$s are born later and migrate from the region close to the midline at the top of the arrow (larger cell) and the cell marked A above and as they migrate out to the periphery of the neural tube along the course of the arrow they pass through a RA-rich zone (released by cell C in the top drawing) and thus they are induced to differentiate into $LMC_L$s by the time they reach the edge (larger cell at the end of the arrow). [From Sockanathan and Jessell (1998).] (C) Cells in another region of the spinal cord, at earlier stages than part (B), require RA for their differentiation. These are in the middle of the spinal cord (two rows of circles) and are ventral interneurons, which are determined as such due to the expression of a variety of homeobox genes including *Dbx1/2*. RA, presumably generated within the spinal cord (arrows), induces *Dbx1/2*. But also involved in the differentiation of these interneurons is sonic hedgehog (shh), which is a ventrally derived protein (arrow coming from ventral region) and bone morphogenetic protein (bmp), which is a dorsally derived protein (arrow coming from dorsal regions). From Pierani *et al.* (1999).

of RA soaked beads instead of somites (Grapin-Botton *et al.,* 1998) and inhibited by disulphiram, an inhibitor of RA synthesis (Gould *et al.,* 1998). A careful analysis of which somites can reprogram the hindbrain gene expression after rostral transplantation revealed a gradual loss of this ability from the first four somites from the 2-somite stage to the 10-somite stage such that at the latter stage only somites caudal to somite 4 have the ability (Itasaki *et al.,* 1996). This gradual loss precisely correlates with the gradual loss of expression of RALDH2 in the first four somites over these same stages (Blentic and Maden, unpublished, 2000).

Later in development, from stage 12 onward, CYP26 is expressed in the developing spinal cord in the dorsal part near the roof plate (Swindell *et al.,* 1999) and is present in human embryonic brain as well (Trofimova-Griffin *et al.,* 2000). Further studies with CYP26 have revealed the very intriguing observation that when is it is overexpressed in P19 cells, then neural differentiation is induced in the absence of RA (Sonneveld *et al.,* 1999). This suggests that one of the CYP26 products (4-*oxo*-RA, 4-OH-RA, 18- OH-RA or 5,6, *epoxy*-RA) might be a neural inducer and the function of CYP26 is not just to metabolize away all-*trans*-RA (but see Section III.G.4).

RALDH2 is eventually expressed in the neural tube, after stage 19 in chicks and day 12.5 in mice embryos, but then only in the motoneurons at limb levels (Zhao *et al.,* 1996; Niederreither *et al.,* 1997; Sockanathan and Jessell, 1998; Haselbeck *et al.,* 1999). Here it plays an important role in generating the "hot spots" of RA synthesis in the cervical and lumbar eminences where the limb buds will grow out (McCaffery and Drager, 1994b) and in inducing the differentiation of one of the classes of motoneurons themselves, the lateral motor column neurons (Sockanathan and Jessell, 1998). In this location RA, generated by RALDH2, seems to act both as a proliferative agent and a differentiative agent as it increases the number of ventral neural tube progenitor cells, thereby increasing the number of motoneurons. This increase in motoneurons is mimicked by a RXR agonist and not a RAR agonist, suggesting the involvement of the RXRs in this process. The crucial role of RALDH2 was demonstrated by virally misexpressing RALDH2 in the thoracic cord (where neither RALDH2 nor lateral motor column neurons are normally present) and the result was that lateral motor columns (LMCs) were generated at nonlimb levels. Furthermore the specific LMCs that were generated were not from cells themselves expressing RALDH2, but adjacent cells, suggesting that there is a paracrine inductive event occurring which involves one cell generating RA from RALDH2 and an adjacent cell being induced to form specific subsets of motoneurons (Fig. 2B).

In addition, there is another class of neurons in the spinal cord, the interneurons, subsets of which are classified by the expression of various homeodomain proteins. RA or retinol induces the appearance of *Dbx1, Dbx2, Evx1/2,* and *En* expressing interneurons in explants of naïve neural plate (Pierani *et al.,* 1999). Because the neural tube does not, at this time point, express RALDH2, then there must be

another retinaldehyde dehydrogenase present that can metabolize retinol into RA and perform this function of inducing subsets of interneurons (Fig. 2C).

The regions of the nervous system that have been identified in the zymography assays (see Section III.A) as synthesizing RA obviously contain RALDHs. In fact RALDH2 has been identified by this methodology in the choroid plexus, meninges of the spinal cord, olfactory bulb, and anterior pituitary (Yamamoto *et al.,* 1996; McCaffery and Drager, 1995) and RALDH3 in the LGE (Li *et al.,* 2000). RALDH3 is also expressed in the ventral retina (see above) and in the otic vesicle (Mic *et al.,* 2000).

A final and fascinating location for the RALDH1 enzyme was found in the adult mouse brain, in the axons of the dopaminergic neurons of the striatum and substantia nigra (McCaffery and Drager, 1994a). This suggests not only an involvement of RA in the maintenance of axonal functioning in this system, but that a retinoic acid generating enzyme can be transported down an axon to generate RA and hence act at a considerable distance from the cell body. This is of relevance to the developing nervous system because RALDH1 is localized during development to cells of the ventral mesencephalon of the mouse embryo from day 9 onward (Haselbeck *et al.,* 1999). This is the origin of the dopaminergic neurons of the substantia nigra and therefore RA may be involved in the differentiation of this part of the brain.

### 2. Alcohol Dehydrogenases

Two classes of enzymes have been implicated in the oxidation of retinol to retinal. One is the cytosolic medium-chain ADHs and the other is the microsomal short-chain alcohol dehydrogenases (SDRs) (Duester, 1996). Enzymes from both groups are able to oxidize retinol *in vitro,* but the relative role of the different enzymes *in vivo* is still unknown. One way of examining their possible roles *in vivo,* and specifically in neural development, is to ask whether they are expressed in neural tissues.

With regard to the ADHs, there are five classes of this enzyme type in humans, but the mouse has only three, namely, I, III, and IV (Haselbeck *et al.,* 1999; Zgombic-Knight *et al.,* 1995) and of these three, only class IV correlates with the above measurements of endogenous RA in the early embryo (Ang *et al.,* 1996) (Figs. 1A–C). However, ADH-IV has an expression that closely matches that of RALDH2 in that it is expressed in the mesoderm and not the neural tube (Ang and Duester, 1997), thus there does not seem to be a neural ADH in addition to the lack of a neural RALDH.

In contrast, one of the SDRs, a retinol dehydrogenase that has the ability to oxidise 9-*cis*-retinol, but not all-*trans*-retinol is expressed in various parts of the developing nervous system of the mouse embryo such as the dorsal neural tube, the yentral floor plate, the isthmus, and the cranial and spinal ganglia (Romert *et al.,* 1998). However, 9-*cis*-RA has not been identified as an endogenous component of embryos so the significance of this enzyme is not clear. Finally, another SDR,

retinol dehydrogenase type 1 (RODH1), is expressed in cultured cervical ganglion neurons (Chandrasekaran *et al.,* 2000), but its distribution in the embryo is not known.

## E. Cells Synthesizing RA in the Nervous System

The above detection of RALDH1 in the axons of dopaminergic neurons (McCaffery and Drager, 1994a) clearly suggests that neurons can make RA, for whatever function. This is also true of motoneurons that express RALDH2 (Haselbeck *et al.,* 1999; Niederreither *et al.,* 1997; Sockanathan and Jessell, 1998; Zhao *et al.,* 1996) and cellular retinol binding protein type I (CRBPI) (Maden *et al.,* 1990) and cervical ganglion neurons which express RODH1, RALDH1, and RALDH2 (Chandrasekaran *et al.,* 2000).

On the other hand, an interesting alternative that has been previously hypothesized is that it is the glial cells which make the RA, which is then transferred to the neurons and the neurons take it up for their own use in much the same way that some Schwann cell factors operate. This idea came from experiments on dissociated cultures of embryonic rat spinal cords (Wuarin *et al.,* 1990). These authors found that in such cultures, both retinol and RA promote the survival and differentiation of neurons. However, in pure neuronal cultures retinol has no effect, only RA is effective, suggesting that retinol is converted to RA by the glial cells. Using cortical neurons or astrocytes they showed that when retinol was added to these cultures RA could only be detected in the medium from astrocytes, not in the medium from neurons. There are two other similar cases. In the cells of the retina, the glial cells known as Muller cells can convert retinol to RA and, again, it is suggested that the adjacent amacrine neurons which contain cellular retinoic acid binding protein (CRABP) take up and use this locally synthesized RA (Edwards *et al.,* 1992). Secondly, in the lateral ganglionic eminence it is specifically the glial cells that contain CRBPI and when cultured these glial cells generate and release RA into the culture medium (Toresson *et al.,* 1999).

Another cell type found to contain relatively high levels of RALDH2 is the ependyma derived choroid plexus (Yamamoto *et al.,* 1996) and the neural crest-derived meninges (McCaffery and Drager, 1995).

Thus it seems that neurons, glia, ependyma, and meninges can synthesize RA, but whether they do so or not depends on their location and developmental age in the CNS.

## F. Binding Proteins and Receptors

The retinol binding protein CRBPI and the retinoic acid binding proteins CRABPI and CRABPII are cytoplasmic proteins involved in the uptake, sequestering, and

metabolism of their respective ligands, retinol and RA (Napoli, 1994). They are present in the CNS with intriguing distributions.

### 1. CRBPI

In the amphibian and chick embryos, CRBPI protein has been localized to a specialized region of the developing neural tube, the ventral floor plate, using an antibody raised against rat CRBPI (Hunter *et al.,* 1991; Maden and Holder, 1991; Maden *et al.,* 1989b). The floor plate is an important organizing region consisting of radial glial cells and is responsible for establishing the dorsoventral pattern of neuronal specification and neurite outgrowth within the cord. In the developing mammalian CNS, however, the same antibody recognizes the early differentiating motoneurons as they are specified (Maden *et al.,* 1990) and then the ventral roots of the spinal cord, adding to the evidence in favor of a role for retinoids in motoneuron specification (see Section III.D.1). The mRNA for CRBPI is expressed in this location in the mammalian embryo, as well as in the ependyma of the later spinal cord and the roof plate (Perez-Castro *et al.,* 1989; Ruberte *et al.,* 1993). Earlier in neural development, however, at the early somite stage of the mouse embryo CRBPI mRNA is more widely expressed in the neural tube from posterior levels up to the hindbrain, but expression is very low in the forebrain (Ruberte *et al.,* 1991). Thus in mammalian embryos CRBPI is expressed widely in the neural tube early on, and becomes gradually restricted during development to motoneurons.

In the developing brain CRBPI is found in the lateral ganglionic eminence (Toresson *et al.,* 1999), the lamina terminalis (Maden *et al.,* 1990), the corpus striatum, hypothalamus, olfactory tubercle, mesencepahlic nucleus of the trigeminal, paraventricular nucleus of the thalamus, the Purkinje cell layer of the cerebellum, most motor nuclei, and in the choroid plexus (Ruberte *et al.,* 1993; Macdonald *et al.,* 1990). CRBPI is also expressed in the developing eye both throughout the neural retina and the pigmented retina layers (Dolle *et al.,* 1990; Perez-Castro *et al.,* 1989), is abundant in the anterior pituitary (Dolle *et al.,* 1990) and is found in the olfactory ensheathing cells of the olfactory system (Gustafson *et al.,* 1999).

### 2. CRABPI

CRABPI has a more restricted distribution than CRBPI. CRABPI is present in a population of interneurons in the developing chick and mammalian neural tube known as the commissural neurons (Dencker *et al.,* 1990; Maden and Holder, 1991; Maden *et al.,* 1989a, 1989b, 1990; Momoi *et al.,* 1989; Perez-Castro *et al.,* 1989; Ruberte *et al.,* 1992, 1993; Shiga *et al.,* 1995; Vaessen *et al.,* 1990), which are located in the mantle layer of the intermediate region of the neural tube and send their axons ventrally toward the ventral floor plate.

In the developing brain CRABPI is not initially expressed in the forebrain, but is expressed in a group of neurons in the roof of the midbrain, which will become

the mesencephalic trigeminal nucleus (Leonard *et al.,* 1995; Maden *et al.,* 1990, 1991, 1992a, Dencker *et al.,* 1990; Perez-Castro *et al.,* 1989; Ruberte *et al.,* 1992, 1993; Vaessen *et al.,* 1989). Later in development (day 15.5 mouse), however, CRABPI is expressed in the LGE (Garel *et al.,* 1999), as is CRBPI (see Section III.F.1). In the developing hindbrain of mammalian embryos CRABPI shows an interesting rhombomere specific expression pattern. By day 9.5 of mouse development CRABPI protein and mRNA is not expressed in rhombomere 1, expressed lightly in rhombomere 2, not in rhombomere 3, then heavily expressed in rhombomeres 4, 5, 6, and 7 (Leonard *et al.,* 1995; Maden *et al.,* 1992a). This pattern is preceded in development, at day 8 when the CNS is just forming, by a single band of CRABPI labeling, which is just posterior to the preotic sulcus. This band of CRABPI expression marks the presumptive posterior rhombomeres of the hindbrain from rhombomere 4 to rhombomere 7 (Leonard *et al.,* 1995; Maden *et al.,* 1992a). Since this is the region that is missing in embryos deprived of RA altogether (see Section IV.A) then RA, acting via CRABPI, may play a role in the specification of this posterior hindbrain region.

The differential distribution of CRABPI in the hindbrain rhombomeres has proved of value in identifying individual rhombomeres in mutant mice, such as *kreisler* (McKay *et al.,* 1994) and *Hoxa-1* null mice (Mark *et al.,* 1993). In the chick embryo this rhombomere specific expression throughout the thickness of the neuroepithelium is not found. Instead there is expression of CRABPI in individual reticulospinal neurons as they differentiate (Maden *et al.,* 1991).

The neural crest also expresses CRABPI and in the hindbrain streams of neural crest migrate out from the rhombomeres. In the mouse embryo the expression levels of CRABPI in the neural crest streams reflect the expression levels of the rhombomeres from which the streams originate (Maden *et al.,* 1992a). Thus the stream of crest from rhombomere 2 lightly expresses CRABPI and gives rise to the lightly expressing mesenchyme of the mandibular arch. The heavily expressing remaining streams from rhombomeres 4 and 6–7 give rise to heavily expressing crest and mesenchyme of the remaining branchial arches. The cranial nerves and ganglia that derive from the neural crest in the hindbrain also express CRABPI (Ruberte *et al.,* 1991).

The neural crest migrating from the spinal part of the neural tube also expresses CRABPI and it thus serves as a very useful marker for studies of the migration routes and the differentiated products of this cell type (Dencker *et al.,* 1990; Leonard *et al.,* 1995; Maden *et al.,* 1989a, 1990, 1992a). These neural crest derivatives in the trunk, which all express CRABPI strongly, are dorsal root ganglia, Schwann cells, sympathetic ganglia, and enteric ganglia.

In other CNS derivatives such as the eye, whereas CRBPI was expressed throughout the retina and lens, CRABPI is only expressed in the center of the neural retina and spreads out as differentiation of ganglion cells proceeds (McCaffery *et al.,* 1993). CRABPI is also expressed by the olfactory axons (Gustafson *et al.,* 1999).

In *Xenopus* a CRABP has been cloned that has properties of both CRABPI and CRABPII and whose size is twice that of its murine counterparts (Ho *et al.,* 1994). During neural development stages this CRABP is present in an anterior domain *and* a posterior domain. The anterior domain consists of expression in the developing telencephalon, then a nonexpressing diencephalon region, followed by a strongly expressing mesencephalon and rhombencephalon region. The posterior domain is in the tail bud and the cranial neural crest also expresses this xCRABP. In contrast, a different CRABP of normal size (15 kDa) has also been cloned in *Xenopus* by Dekker *et al.* (1994). It has a surprisingly similar expression pattern—an anterior (light expression in the forebrain and midbrain, strong expression in the hindbrain) and a posterior (tail bud) domain, as well as the cranial neural crest. RA treatment of embryos enhanced the anterior domain and repressed the posterior domain.

## 3. CRABPII

In the early mouse embryo CNS, CRABPII transcripts are more widespread than those of CRABPI. As the neural tube forms at early somite stages when there is a small band of CRABPI expression in the presumptive hindbrain from rhombomeres 4–6, CRABPII expression begins slightly further posteriorly and is not tightly localized to a band as its expression extends over the length of the embryo and gradually diminishes with no clear posterior boundary (Leonard *et al.,* 1995; Lyn and Giguere, 1994; Ruberte *et al.,* 1992). Thereafter CRABPII also extends rostrally into the midbrain and forebrain and a rhombomere specific expression pattern develops as in the case of CRABPI. Rhombomeres 2, 4, and 6 are the most intensely labeled, rhombomere 5 the least intensely labeled, and the remaining rhombomeres labeled to moderate levels. The midbrain expression virtually disappears, whereas the telencephalic vesicles label intensely. The cranial neural crest is also CRABPII positive, including the frontonasal mesenchyme and the crest that fills the branchial arches.

The expression of CRABPII in the spinal part of the neural tube is also not as precisely localized as that of CRABPI because it shows expression throughout the neural tube early on in mouse CNS development and then, according to Ruberte *et al.* (1993), becomes somewhat localized to the presumptive motoneurons of the ventral cord as well as being present in newly formed neuroblasts in the dorsal cord. However, in another study (Maden, 1999b), there was no expression in the motoneurons and instead there was a strong localization of CRABPII mRNA to the alar half of the spinal cord in a curious chevron shape excluding the most dorsal part of the neural tube. CRABPII is also expressed by the trunk neural crest cells during their migration and differentiation and is thus found in the dorsal root ganglia. In the later developing brain CRABPII is expressed in the lateral walls of the diencephalon and the optic stalk, posterior mesencephalon, pontine nuclei, thalamic nuclei, geniculate body, the motor nuclei of the hindbrain, and the choroid plexus (Ruberte *et al.,* 1992, 1993).

Thus it can be seen that the binding proteins display both overlapping and unique distributions during CNS development, which may provide neuronal cells with differing concentrations of RA or specific retinoids and thus allow them to respond differentially.

## 4. RARs and RXRs

In early CNS development of the mouse embryo, RAR$\alpha$ is expressed throughout the neural tube and anteriorly up to the rhombomere 3/4 border, RAR$\beta$ is expressed in a similar domain up to the rhombomere 6/7 border, and RAR$\gamma$ is expressed in the open neural tube and not the closed neural tube (Ruberte *et al.,* 1991). In the chick embryo RAR$\beta$ is similarly expressed, but the anterior border is one rhombomere more anterior, at the 5/6 boundary (Smith, 1994; Smith and Eichele, 1991) and RAR$\alpha$ is expressed from the rhombomere 6/7 boundary caudally (Hoover and Glover, 2000). Thus the early forebrain, midbrain, and anterior hindbrain develop without any RARs, which fits with the observation that there is no endogenous RA in these regions at these stages (see Section III.A–C). However, it seems that there is a weak and ubiquitous expression of RXR$\alpha$ and RXR$\beta$ in mouse embryos (Dolle *et al.,* 1994) and so these two receptors may be expressed in the developing anterior CNS. The fact that there is a boundary between RAR$\beta$ expression in the closed neural tube and RAR$\gamma$ in the open neural tube suggests a role for these receptors and RA itself in the process of neurulation and it is therefore significant that the expression levels of these receptors be down-regulated in a mouse mutant, *curly tail,* which displays neural tube defects (Chen *et al.,* 1995).

There are also significant variations in isoform distribution (Mollard *et al.,* 2000). RAR$\alpha$1 transcripts are distributed along the spinal cord and decreasing in the hindbrain, whereas RAR$\alpha$2 transcripts are expressed much more strongly up to the level of rhombomere 7 and then in rhombomere 4. The RAR$\beta$1/3 and RAR$\beta$2/4 isoforms are differentially distributed within the dorsoventral axis of the CNS. In the hindbrain RAR$\beta$1/3 is present in the alar plate and ventral horns, whereas $\beta$2/4 is prominent in the ventricular layer. In the spinal cord RAR$\beta$1/3 is prominent dorsally, whereas $\beta$2/4 is more evenly distributed and only $\beta$2/4 is found in the meninges.

Later in development (from day 11.5) RXR$\gamma$ is coexpressed along with RAR$\beta$ in two parts of the forebrain, the dorsal hypothalamus and the corpus striatum (Dolle *et al.,* 1994), and specifically in the LGE, RAR$\alpha$, RAR$\beta$, and RXR$\gamma$ are expressed (Garel *et al.,* 1999). RAR$\alpha$, specifically the RAR$\alpha$2 isoform, is also expressed in the corpus striatum, pons, pallidum, and choroid plexus (Mollard *et al.,* 2000). In the chick RXR$\gamma$ localizes to specific regions such as the mesencephalic nucleus of the trigeminal and the cerebellar plate of the hindbrain (Hoover *et al.,* 2000). RXR$\gamma$ is expressed in the neural retina of the eye in both mouse (Dolle *et al.,* 1994) and chick (Hoover *et al.,* 2000) where it specifically localizes to photoreceptors. In

the developing mouse and chick spinal cord RAR$\beta$ becomes localized to a subset of the developing motoneurons (Rubert *et al.,* 1993; Muto *et al.,* 1991), specifically the $\beta$1/3 isoform (Mollard *et al.,* 2000) at least in the hindbrain motoneurons, along with the coexpression of RXR$\gamma$ (Dolle *et al.,* 1994). As the cervical and lumbar enlargements develop into the "hot spots" of RA synthesis, RAR$\beta$ expression intensifies in these regions and is down-regulated in the intervening segments (Colbert *et al.,* 1995; Rubin and La Mantia, 1999), observations which all accord with the role of RALDH2 and RA in motoneuron differentiation (see Section III.D.1).

Concerning the neural crest, RAR$\alpha$ is expressed in the migrating neural crest at a higher level than the other RARs and it is subsequently expressed in the anterior branchial arches of the mouse embryo (Ruberte *et al.,* 1991) and weakly and uniformly over the facial region (Osumi-Yamashita *et al.,* 1990). RAR$\beta$ is expressed in the neural crest derived mesenchyme around the developing eyes and in the frontonasal mesenchyme and absent from the maxillary process, present in the caudal half of the mandibular arch and absent from the rest of the arches (Ruberte *et al.,* 1990). RAR$\gamma$ is expressed throughout the frontonasal and branchial arch mesenchyme, which will give rise to facial cartilages and bones (Osumi-Yamashita *et al.,* 1990; Ruberte *et al.,* 1990). In the chick embryo RXR$\gamma$ is a specific marker for neural crest and its subsequent differentiated products such as the sensory peripheral nervous system (Rowe *et al.,* 1991) and RXR$\alpha$ is expressed at lower levels (Hoover and Glover, 2000). RAR$\beta$, like the mouse embryo, is expressed by a subset of chick neural crest cells and in the anterior facial primordia particularly in the mesenchyme around the eyes. The border between expressing and nonexpressing cells is between the maxillary and mandibular primordia (Rowe *et al.,* 1992; Smith, 1994).

In the *Xenopus* embryo there is an interesting reciprocal distribution between two isoforms of RAR$\gamma$, with RAR$\gamma$1 being expressed in the anterior end of the embryo including the brain and neural crest in the branchial arches, whereas the RAR$\gamma$2 isoform is expressed at the tail end of the embryo including the floor plate of the posterior spinal cord (Pfeffer and De Robertis, 1994). The levels of most of the other receptors that have been cloned in *Xenopus* drop sharply before gastrulation and so would not be expressed in the CNS (Blumberg *et al.,* 1992).

In zebrafish embryos, RAR$\gamma$ is strongly expressed in the neuroepithelium of the hindbrain with an anterior border corresponding to the region of rhombomere 6/7. RAR$\gamma$ is expressed up to the midbrain/hindbrain border in the ventral part of the neural tube with the highest transcript level being in the posterior hindbrain (Joore *et al.,* 1994). This receptor is also expressed in the head mesenchyme, which is of neural crest origin. Interestingly, in the zebrafish there are not three RXRs as might be expected, but five—$\alpha$,$\beta$,$\gamma$, and two novel ones, $\delta$ and $\varepsilon$ (Jones *et al.,* 1995). The two novel RXRs do not bind 9-*cis*-RA, although we do not yet know anything about their expression patterns.

## G. Knockouts and Overexpression Studies

### 1. Binding Proteins

In the light of the binding protein, enzyme, and receptor distribution in the nervous system described above we might expect a dramatic CNS phenotype from knockout studies. In fact, the contrary is the case as most such mutants are perfectly normal. Thus CRABPI null mutants, CRABPII null mutants, and CRABPI/CRABPII double null mutants are not only normal in their CNS, but in the rest of the body too except for an extra postaxial digit (Fawcett *et al.,* 1995; Gorry *et al.,* 1994; Lampron *et al.,* 1995). CRBPI null mutants are also normal in their CNS development (Ghyselinck *et al.,* 1999).

However, overexpression of the *Xenopus* CRABP produced typical RA-induced defects: anterior abnormalities such as reduced or fused eyes, strongly reduced forebrain and midbrain, loss of hindbrain segmentation, and posterior defects such as kinked or shortened tails (Dekker *et al.,* 1994) (defect 2, Table II). Furthermore the expression of *Hoxb-4* and *Hoxb-8* was enhanced to the same degree as RA treatment, suggesting a role for this xCRABP in the control of AP patterning in the *Xenopus* CNS.

### 2. Knockouts of Receptors

Most of the receptor knockouts produce normal progeny as well; RAR$\alpha$, RAR$\gamma$, RXR$\beta$, and RXR$\gamma$ null mutants all develop a normal nervous system (Kastner *et al.,* 1995; Chiang *et al.,* 1998). However, several mutants have subtle defects in the adult brain perhaps reflecting developmental abnormalities. These include RAR$\beta$/RXR$\beta$, RAR$\beta$/RXR$\gamma$, and RXR$\beta$/RXR$\gamma$ double mutants, which have locomotor defects due to a dysfunction of the dopamine signaling pathway (Krezel *et al.,* 1998), and RAR$\beta$ and RAR$\beta$/RXR$\gamma$ mutants, which have impaired spatial learning and memory defects caused by defective hippocampal functioning (Chiang *et al.,* 1998).

Of the single gene knockouts, only the RAR$\beta$ and RXR$\alpha$ knockouts have any embryonic defects at all related to the CNS and these are primarily ocular defects. One report reveals that one RAR$\beta$ null mouse embryo had fused proximal portions of the glossopharyngeal and vagus nerves (cranial nerves IX and X) (Luo *et al.,* 1995), but this defect did not appear in another study (Ghyselinck *et al.,* 1997) (see below). However, both reports showed ocular defects in the form of an abnormal retrolenticular mass of pigmented tissue adherent to the lens, a folded retina and cataracts. The RXR$\alpha$ null mutants also had ocular defects in which the ventral retina was shortened and the anterior segment of the eye was malformed (abnormal cornea, unfused eyelids, abnormal lens, abnormal conjunctiva) (Kastner *et al.,* 1994a). Widespread eye defects including those of the anterior eye as just described, malformation of the mesenchymal structures such as the sclera,

TABLE II
Summary of the Effects on the CNS of Either Altering RA Levels or Disturbing the RA Signaling Systems

| Defect no. | Cause | CNS phenotype | Reference |
|---|---|---|---|
| 1 | Normal | E FB MB **1 2 3 4 5 6 7** SC | |
| 2 | XCRABP overexpression in *Xenopus* | FB MB – – – – – – – SC | Dekker *et al.* (1994) |
| 3 | RAR$\alpha$/RAR$\gamma$ double knockouts in mouse | E FB MB **1 2 3 4 5 6/7** SC | Dupe *et al.* (1999) |
| 4 | RAR$\alpha$1 dominant negative overexpression in *Xenopus* | E F B M B **1 2 3 4 6 7** SC | Blumberg *et al.* (1997) |
| 5 | RAR$\alpha$1 functional overexpression in *Xenopus* | MB **1 2 3 4 5 6 7** SC | Blumberg *et al.* (1997) |
| 6 | RAR$\alpha$2 overexpression in *Xenopus* | E FB MB **1 2 3 4 6 7** SC | Kolm *et al.* (1997) |
| 7 | RAR$\beta$ overexpression in *Xenopus* | E FB MB **3334445** SC | van der Wees *et al.* (1998) |
| 8 | RALDH2 knockout in mouse | E FB MB **1 2 3** SC | Niederreither *et al.* (2000) |
| 9 | CYP26 overexpression in *Xenopus* | E FB MB **1 2 1 2 3 4 5** SC | Holleman *et al.* (1998) |
| 10 | RA deficiency in rat | E FB MB **1 2 3** – – – – SC | White *et al.* (2000) |
| 11 | RA deficiency in quail | E FB MB **1 2 3** SC | Maden *et al.* (1996) |
| 12 | Excess RA, posteriorization | **1 2 3 4 5 6 7** SC | Durston *et al.* (1989) |
| 13 | Excess RA, loss of anterior hindbrain | E FB MB **4 5 6 7** SC | Morriss-Kay *et al.* (1991) |
| 14 | Excess RA, transformation of anterior hindbrain | E FB MB **1 4 5 4 5 6 7** SC | Marshall *et al.* (1992) |
| 15 | Excess RA, loss of rhombomere boundaries, in chick | E FB MB **1 2 3** – – – – SC | Nittenberg *et al.* (1997) |

*Notes:* Each effect is described in the text and summarized here symbolically for comparisons to be drawn. Of the 14 effects, 12 of them involve the hindbrain, demonstrating how sensitive this part of the CNS is to RA perturbations. Symbols: E = eyes; FB = forebrain; MB = midbrain; 1–7 = individual rhombomeres; SC = spinal cord; – = tissue present but rhombomere boundary lost; FB MB = reduced size; FB MB = increased size; 5 = increased size; **6/7** = fused rhombomeres.

shortened ventral retina, retinal dysplasia, microphthalmia, and postnatal retinal degeneration are all seen seen when double RAR null mutants are created such as RAR$\alpha/\gamma$ or RAR$\beta/\gamma$ (Ghyselinck *et al.,* 1997; Grondona *et al.,* 1996; Lohnes *et al.,* 1994) or double RAR/RXR mutants such as RAR$\gamma$/RXR$\alpha$ (Kastner *et al.,* 1997). Interestingly, the ventral retina thus seems to be a particularly RA sensitive region—it can be missing in certain knockout combinations. RA excess duplicates the ventral retina, and RA deficiency prevents its development (see Section IV.A.3).

A CNS defect seen in RAR$\alpha/\gamma$ double mutants was a persistent opening of the hindbrain leading to neuroepithelial degeneration (Lohnes *et al.,* 1994), but fully penetrant hindbrain phenotypes were seen in RAR$\alpha$/RAR$\beta$ double mutants (Dupe *et al.,* 1999). In these mutants there were ectopic otic vesicles (also seen in RA-deficient rat embryos; White *et al.,* 2000), the glossopharyngeal and vagus nerves were fused and projected aberrantly, the hypoglossal nerve was not fasciculated, and there was a fusion of the third and fourth branchial arches. Within the hindbrain the domain of *Kreisler* (normally rhombomeres 5 and 6) was expanded, the rhombomere 5 stripe of *Krox-20* was expanded and there was ectopic *Hoxb-1* expression. This was interpreted as an expansion of rhombomere 5 and a fusion of rhombomeres 6 and 7 (Table II, defect 3).

Neural crest abnormalities are seen in double null mutants: periocular mesenchymal abnormalities in RAR$\beta/\gamma$ mutants (Ghyselinck *et al.,* 1997; Grondona *et al.,* 1996); abnormalities of the craniofacial and branchial arch skeleton in all combination mutants (Ghyselinck *et al.,* 1997; Lohnes *et al.,* 1994); abnormalities of the thyroid, parathyroid, and thymus glands (derived from the hindbrain neural crest) in RAR$\alpha/\gamma$ and RAR$\alpha/\beta$ mutants (Mendelsohn *et al.,* 1994); and some heart defects (aorticopulmonary septation and aortic arch abnormalities) due to defective cardiac neural crest in RAR$\alpha/\gamma$ and RAR$\alpha/\beta$ mutants (Mendelsohn *et al.,* 1994). These defects thus confirm the role of the RARs in neural crest migration and/or survival.

### 3. Overexpression of Receptors

In the same way that overexpression of CRABP produced a phenotype whereas the knockout did not, overexpression of receptors has generated clear phenotypes within the CNS. Three experiments have been performed, each in *Xenopus* embryos, where the receptors have been overexpressed either as functioning receptors and thereby increasing the amount of RA signaling that occurs or overexpressed as dominant negatives and thereby decreasing the amount of RA signaling. Each experiment has revealed effects on anteroposterior patterning, particularly in the hindbrain.

Blumberg *et al.* (1997) injected either a dominant negative or a constitutively active RAR$\alpha$1 receptor into the *Xenopus* embryo to assess the effects of increasing or decreasing retinoid signaling on AP patterning. The dominant negative receptor led to the anteriorization of the embryos—expansion of forebrain and anterior midbrain, shorter tails, reduced overall length, and an expansion of forebrain marker gene domains. The genes used as markers were *Otx2,* which expanded its domain, *engrailed-2* and *Krox-20,* which both underwent a posterior shift, and *Xlim-1,* which being a posterior marker underwent a down-regulation. Thus the CNS had been anteriorized (Table II, defect 4). Conversely, the constitutively active receptor phenocopied RA treatment and led to a decrease in the *Otx2* domain (reduced forebrain size) and a decrease and anterior shift in the *engrailed-2* and *Krox-20* domains. Thus the CNS had been posteriorised (Table II, defect 5). Similarly,

Kolm *et al.* (1997) overexpressed a RAR$\alpha$2 mutated receptor, which produced a similar, though less strong phenotype than the RAR$\alpha$1 mutation described above. *Hoxd-1* expression was eliminated, the posterior *Krox-20* stripe in rhombomere 5 was eliminated, but *En-2* and *Otx2* were unaffected as was the posterior gene *Hoxb-9* (Table II, defect 6).

Van der Wees *et al.* (1998) injected a dominant negative RAR$\beta$ construct and obtained different results, although the hindbrain was still affected. Presumably the different results reflects the different target genes that RAR$\beta$ activates compared to RAR$\alpha$. The length of the hindbrain was reduced and the rhombomere boundaries were less obvious. There were ectopic Mauthner neurons in rhombomeres 5 and 6 (normally they are only found in rhombomere 4), ectopic *Krox-20* expression in rhombomere 6, *Hoxb-3* was posteriorized and *En-1* and *Hoxb-1* were normal. They interpreted the phenotype as one in which all the rhombomeres were partially converted to a rhombomere 3, 4, or 5 phenotype and therefore the anterior hindbrain had been posteriorized and the posterior hindbrain anteriorized (Table II, defect 7).

## 4. Enzymes

Knockouts of the alcohol dehydrogenase ADH-IV produces normal embryos (Deltour *et al.,* 1999), but this enzyme was not expressed in the nervous system anyway (see Section III.D.2). Although RALDH2 is not expressed in the nervous system until quite late in development and is involved in motoneuron development (see Section III.D.1), the knockout of this gene produces a dramatic phenotype, which mimics almost entirely the vitamin A deficient phenotype in the CNS (see Section IV.A), suggesting that this enzyme is not only the major contributor to RA production in the embryo but also that it does indeed contribute to patterning of the hindbrain as in Figs. 1C–E. Thus the mutant embryo is considerably foreshortened with a truncated frontonasal region, the heart is a single dilated tube, the somites are much smaller than normal, and the embryo dies at midgestation (Niederreither *et al.,* 1999). Most surprisingly, considering the lack of expression of RALDH2 in the early neural tube, the hindbrain of these embryos is considerably reduced and is missing the posterior rhombomeres (Niederreither *et al.,* 2000) (Table II, defect 8). Gene expression data with these mutants using rhombomere specific probes revealed, for example, that *Krox-20* (normally expressed in rhombomeres 3 and 5) was missing the rhombomere 5 stripe, *Wnt8A* was abnormal, *Hoxb-1* (normally expresed in rhombomere 4) was missing, *Kreisler* (normallyin rhombomeres 5 and 6) was missing and *Hoxa-4, b-4,* and *d-4* did not extend into the posterior rhombomeres. The posterior branchial arches were missing, the migration rountes of the neural crest were aberrant, and there was extensive neural crest cell death. This phenotype is therefore virtually identical to that produced in vitamin A deficient rat and quail embryos (see Section IV.A) even though RALDH2 is not expressed in the neuroepithelium. This adds considerable wieght to the proposition

that the RA required for hindbrain development comes from the mesenchyme of the somites as described in Fig. 2A.

CYP26, the enzyme that breaks down all-*trans*-RA into supposedly inactive metabolites such as 4-*oxo*-RA and 4-OH-RA, has been overexpressed in P19 cells and *Xenopus* embryos. We would expect the results of this experiment to be equivalent to removing RA. In P19 cells the surprising result was that overexpression of CYP26 allowed the cells to undergo neural differentiation even in the absence of RA (Sonneveld *et al.,* 1999). This suggests that one of the CYP26 products (4-*oxo*-RA, 4-OH-RA, 18-OH-RA or 5,6, *epoxy*-RA) might be a neural inducer and the function of CYP26 is not just to metabolize away all-*trans*-RA. However, in *Xenopus* embryos, overexpressed CYP26 did seem to behave as a RA removal mechanism because it rescued the embryos from RA-induced morphological defects and rescued *Krox-20, Pax6,* and *En-2* expression in the hindbrain (Hollemann *et al.,* 1998). Overexpressing CYP26 alone caused a posterior shift of three hindbrain markers, *Krox-20, Pax6,* and *Hoxb-3* with no positional change in *En-2* (anterior end of the hindbrain) except for a slight increase in the width of the band, *Hoxb-9* (anterior end of the spinal cord) and a slight expansion but no positional change of *Otx2*. There was also a duplication of the trigeminal ganglion, suggesting a respecification of the anterior rhombomeres (Table II, defect 9).

## IV. Effects of Deficiency and Excess of RA on the Developing CNS

### A. Deficiency of RA

#### 1. Dietary Deficiency

If retinoids are an important component of the developing nervous system, then depleting the embryo of vitamin A should result in abnormal CNS development. This indeed is the case as vitamin deficiencies, particularly of vitamin A, were the first dietary means of producing congenital malformations of the embryo. Initial experiments, performed as long ago as the 1930s on pigs (Hale, 1933), resulted in a litter of pigs being born without eyes. Such malformations have, over subsequent decades, been repeatedly seen in similar experiments using rats, rabbits, cattle, and sheep, complemented with observations on humans. The abnormalities in the mammalian CNS include hydrocephalus, spina bifida, anopthalmia, microphthalmia, and retina malformations (Kalter and Warkany, 1959; Warkany, 1945; Wilson *et al.,* 1953). More detailed studies on the precise anatomical defects in these mammalian studies were not, at that time, performed and now that there is a wide spectrum of molecular and neuroanatomical markers available it is clear that some very specific nervous system abnormalities are apparent.

Using rat embryos, Dickman *et al.* (1997) have adminstered a sudden, acute retinoid deficiency during a selected gestational window by dietary means. A deficiency created from embryonic day 11.5 to day 13.5 resulted in an underdeveloped hindbrain, absent cranial flexure, microphthalmia, and foreshortening of the snout and branchial arches. In the eye, the lens showed apoptosis and the retina failed to invaginate to form the optic nerve. There was considerable apoptosis in the neural crest of the frontonasal mass and maxilla, but not the mandible or hyoid. The cranial nerves were defective, again due to extensive apoptosis, and the vagus nerve was absent. The neural tube was reduced in size and cellularity and the brain had reductions in differentiating neuronal populations due to reduced proliferation.

Also using rat embryos, but giving constantly low or absent levels of RA to pregant dams, White *et al.* (1998, 2000) have generated some very precise CNS defects that are remarkably similar to the RALDH2 knockout embryos (see Section III.G.4) and the vitamin A deficient quail embryos (see below). At the lowest level of RA (not a complete absence) there is an absence of the posterior branchial arches, the cranial nerves IX–XII and associated sensory ganglia are absent, there is a loss of hindbrain segmentation caudal to the rhombomere 3/4 boundary (although not necessarily a loss of tissue) as judged by the expression of *Hoxb-1* (not expressed as normal in rhombomere 4), *Krox-20* (rhombomere 3 stripe wider than expected and rhombomere 5 stripe absent), *Hoxb-4,* and *Hoxb-5* are absent from the caudal hindbrain, and the streams of neural crest were narrow or absent (Table II, defect 10).

Chick and quail embryos have also been used for vitamin A deficiency studies and the original observations using such embryos observed a failure of cardiovascular development (Thompson, 1969; Heine *et al.,* 1985; Dersch and Zile, 1993). The nervous system of the deficient quail embryos show three clear defects (Maden *et al.,* 1996; Gale *et al.,* 1999). The first defect is that the posterior part of the hindbrain is completely missing exactly as in the RALDH2 knockouts, but more severe than the vitamin A depleted rat embryos where only the posterior boundaries were missing. Instead of the normal seven rhombomeres there were only three, numbers 1, 2, and 3, which was established using the expression patterns of the genes *Krox-20, Fgf-3, Hoxa-2, Hoxb-1, Kreisler, Pax-2,* and *Fgf-8*. Thus in the absence of retinoids, rhombomeres 4, 5, 6, and 7 failed to develop (Table II, defect 11). Surprisingly, the rostrocaudal length of the deficient 3 rhombomere hindbrain is the same as that of the normal 7 rhombomere hindbrain (Gale *et al.,* 1999) and so there has been a compensation and respecification of anterior hindbrain tissue to fill in the missing gap. Associated with this loss of hindbrain is the loss of all the cranial nerves posterior to the trigeminal nerve.

The loss of this neural tissue comes about very early in development, as the neural tube forms (Maden *et al.,* 1997). Soon after gastrulation, at the 5 somite stage, a band of cells in the mesoderm in the region of the first somite underwent programmed cell death. This was followed 2 hr later by a band of cell death in the neuroepithelium of the prospective hindbrain. Thus the absence of the posterior

hindbrain is partly caused by a highly localized region of cell death occuring as the hindbrain becomes specified at the 7 somite stage, presumably due to the aberrant expression of the genes that are responsible for the regionalization of the anteroposterior axis in the early neural plate.

The second defect in the CNS of the deficient quail embryos is that as differentiation of neurons in the neural tube begins, the axons of the motoneurons do not project into the periphery as one expects and the axon trajectories within the neural tube itself are disorganized and chaotic. The former may be related to the role of RA in motoneuron differentiation (see Section III.D.1) and the latter to the general phenomenon of RA induced neurite outgrowth (see Sections II, IV.B.6, and IV.B.7). Neurites do not grow out from the embryonic vitamin A deficient neural tube in culture either, but when RA is added to the medium then neurities appear (Maden *et al.,* 1998c) confirming that this is a specific effect of retinoid deficiency.

The third defect seen in these embryos is widespread apoptosis in the neural crest, as seen in the depleted rat embryos. This neural crest cell death begins at about stage 14 and affects all neural crest cells throught the embryo whether they have finished migrating or are still migrating. Streams of apoptotic cells coincide with the known pathways of migration of neural crest cells (Maden *et al.,* 1996). Associated with this loss of neural crest and the posterior hindbrain is the absence of the branchial arches posterior to arch 1.

### 2. Antisense Method

A novel technique recently described for generating retinoid deficiency by a nondietary means is to administer antisense oligodeoxynucleotides for retinol binding protein (RBP) mRNA by injecting them into the yolk sac cavity of cultured mouse embryos (Bavik *et al.,* 1996). Such treatment at the 3–5 somite stage results in accumulation of blood islands on the yolk sac due to lack of vitelline vessels, an identical result to that seen in deficient chick and quail embryos (Dersch and Zile, 1993; Thompson, 1969). Exencephaly was produced in the embryos and the eyes were defective in that the lens placode failed to appear and the optic vesicle failed to invaginate. The expression of three genes was investigated. TGF-$\beta$ and *sonic hedgehog* were both down-regulated in oligo-treated embryos whereas there was no effect on *Wnt-3a*.

### 3. Inhibition of RA Synthesis

An additional method of depriving embryos of RA is to inhibit its synthesis with drugs. Citral, for example, is a competitive inhibitor of aldehyde dehydrogenases and inhibits RA synthesis in the mouse (Connor and Smit, 1987; McCaffery *et al.,* 1992) and zebrafish (Marsh-Armstrong *et al.,* 1994) embryo. The result of treating zebrafish embryos with citral for 2 hr at neurula stages is that the ventral half of the eye fails to develop (Marsh-Armstrong *et al.,* 1994). This observation complements

the finding that RA treatment of zebrafish embryos at the early somite stage resuls in bifurcated eyes that have two retinas due to the overgrowth of the ventral part (Hyatt *et al.,* 1992). Treatment of *Xenopus* embryos with citral also produces eye defects (microphthalmia) along with laterally expanded heads, changes in pigmentation, heart defects, and blood pools (Schuh *et al.,* 1993). In chick embryos citral has been used to generate microphthalmia (Abramovici *et al.,* 1978) thereby emphasizing the role of retinoids in eye development. Treatment of mouse embryos with citral at a specific period of development inhibits the olfactory system, another part of the embryo that depends on RA for its development (Anchan *et al.,* 1997) (see Section III.C).

Another drug, disulphiram, inhibits aldehyde dehydrogenases (Marsh-Armostrong *et al.,* 1995; McCaffery *et al.,* 1992; Vallari and Pietruszko, 1982) and so can be used in the same way as citral to deplete the embryo of RA. However, its effects seem to be different in that treatment of zebrafish embryos with disulphiram produced embryos with short tails and wavy notochords, and the effect on the CNS was to shorten the spinal cord (Marsh-Armstrong *et al.,* 1995).

## B. Excess RA

I next describe the effects of excess RA on the CNS. Although it could be argued that teratogenic effects do not tell us anything about endogenous mechanisms, in fact, the effects of RA on embryos have confirmed the results obtained with EC cells on the role of RA in neural differentiation and led to important insights into how the *Hox* genes are controlled within the neuroepithelium.

The teratogenicity of retinoids on the CNS and its derivatives was first recognized in 1953 when Cohlan reported a high level of exencephaly and a low level of eye defects in rat embryos after treating the mothers with vitamin A (Cohlan, 1953). Since that time the many reports on the teratogenicity of retinoids on the CNS have confirmed and extended these findings, which can be summarized as microphthalmia, encephalocoel, exencephaly, spina bifida, and microcephaly. Following exencephaly there is a buildup of dividing neuroblasts in the everted neural folds due to a lack of differenetiation and the cells collect into rosette-like structures (Langman and Welch, 1967; Yasuda *et al.,* 1989). In addition, there are also characteristic defects of the head and neck which can be attributed to effects on the neural crest—hypoplastic maxilla or mandible, microtia, thyroid, and thymus malformations (Alles and Sulik, 1990; Langman and Welch, 1967; Shenfelt, 1972; Sulik *et al.,* 1995; Yasuda *et al.,* 1986). Sadly, the same spectrum of CNS abnormalities is seen in humans after inadvertent administration of 13-*cis*-RA to the embryo (Die-Smulders *et al.,* 1995; Lammer and Armstrong, 1992; Lammer *et al.,* 1985; Rosa *et al.,* 1986).

There are three different effects of RA on patterning within the CNS, which are caused either by differences in concentration (in *Xenopus*) or differences in

stage of treatment (in rat and mouse) or treatment with different RA isoforms (in zebrafish). These are posteriorization, loss of anterior hindbrain, and posteriorization of the hindbrain. In addition there is a fourth effect whereby RA promotes neural differentiation in embryos, which is precisely what one might have expected from the results on EC cells (Section II).

### 1. Posteriorization

Posteriorization of the CNS is the "classical" effect, which is widely observed and is reflected in the fact that the forebrain and eyes are missing, the domains of expression of anterior genes are extinguished, and the domains of expression of posterior genes are expanded. This was first observed by Durston *et al.* (1989) using the *Xenopus* embryo and treating them with RA between the late blastula and early neurula stages. They noted that the embryos failed to develop anterior neural structures such as the forebrain, midbrain, and eyes and that the hindbrain seemed to be expanded to compensate (Table II, defect 12). Identical effects are seen with axolotl embryos (Maden *et al.,* 1992b). This repression of anterior-specific genes such as *Otx2, XCG-1, XAG-1,* and *XA-1,* and the up-regulation of posterior genes such as *Krox-20, XlHbox6, Xhoh.lab1,* and *Xhox.lab2* after RA treatment is a common finding (Cho and De Robertis, 1990; Dekker *et al.,* 1992; Leroy and De Robertis, 1992; Lopez and Carrasco, 1992; Lopez *et al.,* 1995; Pannese *et al.,* 1995; Sive *et al.,* 1990). Further experiments revealed that RA could mimic normal development by generating the correct spatial pattern of expression of two genes, *X1Hbox6* and *XIF6* in isolated explants of *Xenopus* animal caps (Sharpe, 1991). These experiments led to the suggestion that there is an endogenous gradient of RA within the neural plate with a high point at the posterior end, but we now know that this is not the case, as the high point is at the level of the first somite, at least in chicks and mouse (Fig. 1E).

The *Xenopus* embryo has also been valuable in asking whether this posteriorization directly affects the CNS during its specification or earlier on the mesoderm and thus only secondarily on the CNS. To answer this, isolated parts of the embryo have been treated with RA and then recombined or embryos have been treated before or after the induction of the nervous system and the general conclusion from these experiments is that RA acts *both* directly on the CNS and indirectly via the underlying mesoderm (Ruiz i Altaba and Jessell, 1991a, 1991b; Sive and Cheng, 1991; Sive *et al.,* 1990). This explains why the same general effects on anterior neural structures are seen irrespective of whether the embryos are treated with RA before or after neural induction. However, the fact that this posteriorization involving the down-regulation of *Otx2* and induction of *Krox-20* can occur in isolated animal caps (Taira *et al.,* 1997) in the absence of the induction of mesodermal markers such as *X-bra* (Papalopulu and Kintner, 1996), demonstrates that there are endogenous patterning mechanisms involving RA that operate within the early CNS.

This posteriorization has not only been seen in *Xenopus,* but in all vertebrate embryos studied. It occurs in zebrafish after treatment with 9-*cis*-RA rather than all-*trans*-RA (Zhang *et al.,* 1996) and in rat and mouse embryos (Avantaggiato *et al.,* 1996; Cunningham *et al.,* 1994; Simeone *et al.,* 1995). In rat embryos, where the effect is seen after treatment at the late streak stage, there is a progressive loss of anterior structures with increasing length of exposure to RA, exactly as seen in *Xenopus* embryos. In mouse embryos this effect also peaks after treatment at mid-late streak stage and the embryos showed absence of anterior sense organs, reduction of the anterior brain volume accompanied by an increase of the hindbrain mass, and poor differentiation of the neuroepithelium (Simeone *et al.,* 1995). The loss of *Emx1, Emx2,* and *Dlx1* gene expression domains (normally expressed in the forebrain) and the anteriorization of the *Wnt-1, En-1, En-2,* and *Pax-2* (normally expressed more posteriorly in the midbrain and hindbrain) confirmed the morphological observations (Avantaggiato *et al.,* 1996). *Otx2* is considered to be an important gene in determination of the forebrain as a targeted mutation of the gene in mouse leads to the loss of anterior neural structures, a phenotype that is remarkably similar to the effect of RA (Ang *et al.,* 1996). The down-regulation and shift of the expression border of *Otx2* is also seen in the chick (Bally-Cuif *et al.,* 1995) and zebrafish embryo (Joore *et al.,* 1997) and in the latter case *goosecoid* is also down-regulated.

Thus RA induces a stage-specific repatterning of the anterior CNS by altering the genes used to regionalize the CNS. If RA is normally absent from the early anterior neural plate (Fig. 1E) then its presence will induce this aberrant gene expression, for example, the down-regulation of *Otx2,* leading to loss of anterior structures.

### 2. Loss of Anterior Hindbrain

The external phenotype of embryos showing this effect has long been recognized: an abnormally rostral position of the otocyst and a shortened preotic hindbrain (Morriss, 1972). It results from the loss of a section of CNS tissue tissue in the posterior midbrain/anterior hindbrain region (Table II, defect 13) and is a universal teratological finding not only in rat and mouse embryos (Cunningham *et al.,* 1994; Lee *et al.,* 1995; Leonard *et al.,* 1995; Morriss, 1972; Morriss-Kay *et al.,* 1991; Simeone *et al.,* 1995), but also in chick (Lopez *et al.,* 1995; Sundin and Eichele, 1992), *Xenopus* (Lopez *et al.,* 1995; Papalopulu *et al.,* 1991), and zebrafish (Holder and Hill, 1991; Zhang *et al.,* 1996). In *Xenopus,* this effect is seen at a lower dose of RA than that needed for posteriorization; in zebrafish it is seen when using all-*trans*-RA rather than 9-*cis*-RA; and in rat and mouse embryos it is stage dependent—treatment at the foregut pocket stage for loss of anterior hindbrain rather than the late streak stage for posteriorization (Cunningham *et al.,* 1994; Morriss-Kay *et al.,* 1991).

After RA treatment of these embryos, the typical effect on a variety of *Hox* genes is to induce an anterior spread of their expression patterns into the midbrain and forebrain regions where they are not normally expressed and then they retract posteriorly leaving behind an aberrant expression pattern (Conlon and Rossant, 1992). The result is a clear disruption or obliteration of rhombomeric segmentation (Papalopulu 1991; Lopez *et al.,* 1995) and a loss of several rhombomeres (Leonard *et al.,* 1995; Morriss-Kay *et al.,* 1991; Papalopulu *et al.,* 1991; Wood *et al.,* 1994). But what rhombomeres have been lost? Some have suggested that the anterior hindbrain takes on the gene expression characteristics of a single large rhombomere 4 (Conlon and Rossant, 1992; Wood *et al.,* 1994). But the two other characteristic features of this RA treatment are the fusion of the trigeminal ganglion (derived from rhombomere 2 neural crest) and facial ganglion (derived from rhombomere 4 neural crest) and the fusion of the first and second branchial arches (Cunningham *et al.,* 1994; Kessel, 1993; Lee *et al.,* 1995; Leonard 1995; Pratt *et al.,* 1987; Seegmiller *et al.,* 1991). Both of these effects could be brought about by the loss of rhombomere 3, which would result in the two streams of neural crest normally arising as separate entities, one from rhombomere 2 and one from rhombomere 4, coming together to form a fused ganglion and migrating ventrally en masse, thus causing the arches to fuse. In support of this interpretation, the anterior stripe of expression of the *Krox-20* gene, which is normally expressed in rhombomere 3, is absent in RA-treated embryos (Morriss-Kay *et al.,* 1991; Papalopulu *et al.,* 1991; Wood *et al.,* 1994). However, an analysis of neuronal differentiation in RA-treated embryos revealed that there does indeed seem to be a single large rhombomere at the anterior end of the hindbrain and its motoneurons show the characteristic architecture of the rhombomere 4 based facial nerve (Mallo and Brandlin, 1997). Thus the transformation of the anterior hindbrain into a single large rhombomere 4 agrees with both molecular markers (Morriss-Kay *et al.,* 1991; Wood *et al.,* 1994) and neuronal pathways (Mallo and Brandlin, 1997), but this does not result in the coordinated transformation of the trigeminal ganglion to a facial ganglion. There is only a partial fusion of the ganglia caused by aberrant neural crest migration (see below).

In such RA-treated embryos the migration of neural crest cells is either inhibited or misdirected. In mouse embryos treated with 13-*cis*-RA, the neural crest either does not leave the neuroepithelium or remains near it and the eventual abnormalities seen in these mouse embryos were strikingly similar to those reported in humans who have taken Accutane (13-*cis*-RA) including ear and thymus defects, hypoplastic maxilla or mandible and microtia (Pratt *et al.,* 1987; Webster *et al.,* 1986). A similar failure of crest to migrate occurs in the chick embryo (Moro Balbas *et al.,* 1993). In rat embryos treated with retinyl palmitate, preotic neural crest cells have been observed to migrate more slowly and their pathway was shifted more anteriorly (Morriss and Thorogood, 1978) and mis-migration in rat embryos has been directly observed in that DiI-labeled anterior hindbrain crest migrates into the second branchial arch instead of the first arch as normal (Lee *et al.,*

1995). Also, rhombomere 4 neural crest mis-migrated anteriorly to contribute to the trigeminal ganglion instead of populating the facial ganglion as normal. In the chick embryo a similar mis-migration of DiI-labeled crest in two directions from rhombomere 4 has been seen. Misrouting in a rostral direction resulted in an aberrant contribution to the trigeminal ganglion and caused a fusion of the trigeminal and facial ganglia, and misrouting in a caudal direction resulted in completely aberrant facial nerve tracts (Gale *et al.,* 1996).

Thus it may be concluded that the loss of anterior hindbrain is characterized by a loss of rhombomeres 1, 2, and 3, enlagred rhombomere 4, fusion of the trigeminal and facial ganglia, fusion of the first and second branchial arches, and mis-migration of the neural crest (Table II, defect 13).

### 3. Transformation of Anterior Hindbrain

The third phenotype is a curious combination of both 1 and 2 in that the anterior hindbrain is affected and it is posteriorized. In mouse embryos, transgenic animals were generated that contained *LacZ* reporter genes to reveal the endogenous expression of various *Hox* genes (Marshall *et al.,* 1992). *Hoxb-1,* for example, is a gene which in the day 9.5 mouse embryo is restricted to one rhombomere, rhombomere 4, and to the cranial nerve which derives from rhombomere 4, the facial nerve. After treatment of late streak stage embryos with relatively low doses of RA the expression of *Hoxb-1* at first shifts anteriorly (as is typical for *Hox* genes; see Section IV.B.8), but then becomes progressively restricted to two stripes in the hindbrain rather than the normal one. The second stripe is in rhombomere 2. The normal cranial nerve that emerges from rhombomere 2, the trigeminal nerve, is also changed in character and comes to resemble the normal rhombomere 4 nerve, the facial. Thus rhombomere 2 has been transformed into a rhombomere 4 and, in addition, the authors concluded that the intervening rhombomere 3 comes to resemble a rhombomere 5 (Marshall *et al.,* 1992) so the normal sequence of rhombomeres of 1, 2, 3, 4, 5, 6, 7 has been transformed to 1, 4, 5, 4, 5, 6, 7 (Table II, defect 14). Further neuroantomical data have confirmed that a change does also occur in rhombomere 3. Axons of motoneurons in rhombomere 3 normally project anteriorly and exit with the trigeminal nerve from rhombomere 2. After RA treatment these axons now project posteriorly and join the facial nerve to exit in rhombomere 4 (Kessel, 1993). This clearly indicates a change of motoneuron phenotype in rhombomere 3 and would have a significant effect on the motor innervation of the face, an observation of relevance to the appearance of facial palsies in Accutane-exposed children.

The difference between loss of anterior hindbrain and transformation of anterior hindbrain in the mouse is the stage of development that the embryos have reached when treatment is performed (Lee *et al.,* 1995; Simeone *et al.,* 1995; Wood *et al.,* 1994). In the zebrafish embryo the difference is due to dose as the transformation is seen with a slightly lower dose of RA than that used to generate the loss of tissue (Hill *et al.,* 1995) which, remarkably, happens only by changing the treatment dose

from $1.5 \times 10^{-7}$ *M* to $1 \times 10^{-7}$ *M*. In zebrafish and other lower vertebrates there is a unique neuron present in rhombomere 4 called the Mauthner neuron, which has aided these studies because the appearance of ectopic Mauthner neurons in rhombomere 2 is easy to recognize. The duplication of Mauthner neurons is also seen in *Xenopus* embryos (Manns and Fritzsch, 1992).

### 4. Loss of Posterior Rhombomere Boundaries

An unusual phenotype exists in the chick embryo after placing an RA-soaked bead into the neuroepithelium of a stage 10 embryo. This is the loss of the posterior rhombomere boundaries, only rhombomeres 1, 2, and 3 being distinguishable (Nittenberg *et al.,* 1997) (Table II, defect 15). Although widespread loss of rhombomere boundaries is a common observation in the mouse embryo (Morriss-Kay *et al.,* 1991; Wood *et al.,* 1994) the chick result is a selective loss and it is surprisingly similar to the converse effect of removing all the RA from a rat embryo (Table II, defect 10). The genes normally associated with rhombomere 5 were not expressed (*cek-8, Krox-20*) and some *Hox* genes were anteriorized—*Hoxb-4,* normally up to the rhombomere 6/7 border, was anteriorized to the rhombomere 3 border. Subsequently there was a reduction in the number of motoneurons originating from rhombomere 3 and a severe reduction in the number of contralateral vestibulo-acoustic (CVA) neurons that originate in rhombomere 4. The cranial nerves were somewhat disorganized with ectopic rootlets and nerve bridges between some of the nerves, for example, between the trigeminal and facial.

That this abnormal cranial nerve fusion was caused by RA-induced abnormal migration of neural crest cells had been shown by Gale *et al.* (1996) in chicks. In these experiments where RA was injected focally into the hindbrain rhombomeres the loss of CVA neurons had also been seen and was due to the complete down-regulation of *Hoxb-1* in rhombomere 4, which RA caused and it was suggested that this might be the function of this particular *Hox* expression. Subsequent knockouts of this gene confirmed these conclusions as the *Hoxb-1* mutant mice do not have any CVA neurons (Studer *et al.,* 1996; Goddard *et al.,* 1996).

### 5. The Hindbrain—a Summary

The results discussed above in Sections III.G and IV have revealed that whenever the levels of RA or any of its molecular transducers are altered it is always the hindbrain that is disturbed. Overexpression of xCRABP, knockouts of enzymes or RARs, overexpression of RARs, removal or decrease in the supply of RA, administration of excess RA all give hindbrain phenotypes that are drawn together in Table II to emphasize this point. Sometimes the eyes, forebrain, or midbrain can be missing, but in this case the hindbrain is expanded to compensate and it is equally striking that the spinal cord is always normal in terms of its anteroposterior patterning (although later axonal pathways can be disturbed; see Section IV.B.7).

What does this tell us about the patterning of the hindbrain? I have already discussed the idea that the hindbrain develops between an area beginning at the level of somite 1 that generates RA via RALDH2 and an area (the presumptive forebrain and midbrain) that metabolizes RA via CYP26 as shown in Figs. 1D and 1E. The suggestion is that a gradient of RA is generated across the presumptive hindbrain neuroepithelium and different concentrations of RA establish the differential patterns of activity of genes such as *Hox, Krox-20, Kreisler,* etc., according to the classic concepts of positional information (Wolpert, 1969) as shown in Fig. 3A.

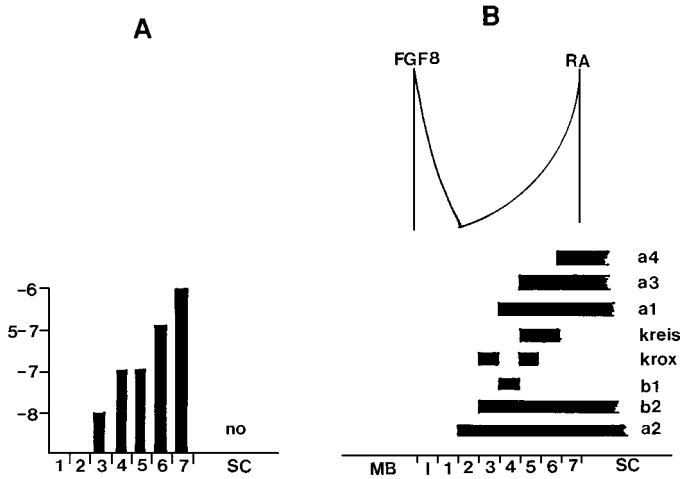



FIG. 3 (A) The effect of increasing doses of RA on marker gene inhibition in the rhombomeres of *Xenopus* embryos. On the vertical axis are the doses of RA administered: $-8 = 10^{-8}\,M$; $-7 = 10^{-7}\,M$; $5-7 = 2.5-5 \times 10^{-7}\,M$; $-6 = 10^{-6}\,M$. Along the horizontal axis the numbers represent the individual rhombomeres of the hindbrain and SC = spinal cord. There were no markers for rhombomere 1 or 2 used. Above a concentration of $10^{-8}\,M$ the marker for rhombomere 3 (*Krox-20*) was inhibited. Above a concentration of $10^{-7}\,M$ the markers for rhombomere 4 (*Hoxb-1*) and rhombomere 5 (*Krox-20*) were inhibited. Above a concentration of $2.5–5 \times 10^{-7}\,M$ the marker for rhombomere 6 (*Hoxb-3*) was inhibited. Above a concentration of $10^{-6}\,M$ the marker for rhombomere 7 (*Hoxb-4*) was inhibited. There was no effect on more posterior spinal cord markers. This clearly shows a graded, concentration-dependent effect of RA on gene inhibition within the hindbrain. Data from Godsave *et al.* (1998). (B) Gene expression in the hindbrain in relation to the gradient of RA from the spinal cord and the gradient of FGF-8 from the isthmus. Along the bottom is a line representing some of the regions of the CNS: MB = midbrain; I = isthmus, a region at the midbrain/hindbrain border; 1–7 = rhombomeres 1–7; SC = spinal cord. Above this line are some examples of gene expression domains showing their anterior borders in the hindbrain coinciding with rhombomere boundaries: a2 = *Hoxa-2;* b2 = *Hoxb-2;* b1 = *Hoxb-1;* krox = *Krox-20;* kreis = *Kreisler;* a1 = *Hoxa-1;* a3 = *Hoxa-3;* a4 = *Hoxa-4*. At the top is the putative gradient profile of RA from the spinal cord diffusing anteriorly into the hindbrain to induce the hindbrain gene expression domains and the profile of FGF-8 diffusing posteriorly from the isthmus into rhombomere 1 to inhibit the expression of Hox genes and thus ensure they cannot be expressed more anteriorly than the rhombomere 1/2 border. (Taken from Irving and Mason, 2000.)

The phenotypes in Table II are interpretable according to just such a hypothesis—too little RA prevents the appearance of genes such as *Kreisler* and *Krox-20* and the posterior hindbrain fails to form; too much RA causes an expansion of the *Hox* gene domains resulting in the formation of too much posterior hindbrain.

A careful study of the effects of excess RA on gene activity in *Xenopus* has highlighted further subtleties both in the concentration dependencies of gene expression domains and exactly what happens to the domains. Godsave *et al.* (1998) gradually increased the dose of RA administered and found that as the concentration was raised from $10^{-8}$ to $10^{-6}$ *M*, there is a stepwise activation of genes in gradually more posterior rhombomeres (Fig. 3B). In addition, for all of the hindbrain genes studied as the concentration was raised they first expanded their domains of expression prior to being extinguished. Therefore RA can can do three things to these genes: (1) switch on in the first place, (2) ectopically induce, and (3) inhibit, according to the particular concentration experienced.

To complete the picture of the hindbrain, it has recently been shown that there is a region at the midbrain/hindbrain border, called the isthmus, which releases fibroblast growth factor 8 into the anterior hindbrain and *inhibits* the expression of *Hox* genes (Fig. 3B) (Irving and Mason, 2000). This explains why rhombomere 1 does not express any *Hox* genes. Thus there is an inhibitor of *Hox* gene expression at the anterior end of the hindbrain and a stimulatory molecule at the posterior end.

## 6. Neuronal Differentiation

The above three effects of RA on development were concerned with patterning of the CNS, but there have also been experiments that concern the role of RA in neural differentiation. These are much closer to the studies on EC cells (Section II), which have identified sequences of gene inductions involved in neural differentiation (Table I) and it is interesting to consider whether the two types of experiment, one on EC cells *in vitro* and the other on embryos, produce the same result. Although the actual downstream genes studied have been somewhat different, the overall result is comfortingly identical: RA induces neuronal differentiation both *in vivo* and *in vitro* and the inhibition of RA signaling or RA production inhibits neuronal differentiation.

For example, neurofilament staining of the RA-deficient quail embryo is strongly down-regulated compared to the normal embryo (Maden *et al.,* 1996), attesting to the decrease of neuronal differentiation *in vivo* in the absence of RA. All of the other experiments of this type have been performed on *Xenopus,* showing how valuable this model system is. Thus Blumberg injected a dominant negative RAR$\alpha$ into *Xenopus* embryos and caused the reduction or ablation of the N-tubulin positive primary neurons, which normally form in three longitudinal stripes in the early embryo (Blumberg *et al.,* 1997). When the embryos reached the tadpole stage they were completely unresponsive to touch stimuli. The same result was obtained by Sharpe and Goldstone (1997) who also showed the converse result that when

the embryos were injected with RAR$\alpha$ along with RXR$\beta$ (together they form the active heterodimer) ectopic primary neurons were generated lying adjacent to the neural tube.

Experiments using RA itself, but performed on animal caps of *Xenopus* embryos, gave the same result. The animal caps had been injected with *noggin* and *XASH-3* mRNA to promote neurogenesis and mimic the normal embryonic situation and then RA treatment promoted N-tubulin expression (Papalopulu and Kintner, 1996). This promotion of neuronal differentiation was suggested to be due to the posteriorization effect of RA (see Section IV.B.1) because when animal caps were treated with *noggin, XASH-3,* and RA they became posteriorized as assayed by the down-regulation of the anterior markers *Xotx2* and *XBF1* and the up-regulation of the posterior marker *Hoxb-3,* and that they were linked was shown by the fact that the ability to posteriorize the embryo is lost at the same time as the ability to induce N-tubulin expression. The effect of RA on whole embryos has been investigated, again in *Xenopus,* and it was observed that, as in animal caps, N-tubulin is up-regulated (Franco *et al.,* 1999). Conversely, a RAR antagonist blocked N-tubulin expression. Upstream of N-tubulin is a gene *X-MyT1,* which is also increased by RA and decreased by the RAR antagonist, and upstream of that is a proneural gene *X-ngnr-1,* which behaves in exactly the same way. In this manner, Franco *et al.* (1999) elaborated a cascade of genes involving *X-delta-1,* another neurogenic gene; *Gli3,* which induces primary neurogenesis; and *Zic3,* which inhibits neurogenesis (Fig. 4). A similar elaboration of a cascade by Gomez-Skarmeta *et al.* (1998) has identified a gene called *Xiro,* which acts upstream of *XASH-3* and *X-ngnr-1,* and is up-regulated by RA. These analyses have revealed that RA acts after the initial induction of neural tissue from mesoderm by molecules such as noggin, follistatin, and chordin, which generate neural tissue of an anterior character, expressing genes such as *Otx2.* Then RA acts to posteriorize this anterior neural tissue by inducing posterior patterning genes (see earlier) and at the same time neurogenic genes such as *Xiro, XASH-3, X-ngnr-1,* and ultimately N-tubulin. These interactions are summarized in Fig. 4.

### 7. Neuronal Pathfinding

After neuronal differentiation has occurred, RA seems to have continuing effects on neurite outgrowth and the pathways that neurons chose to navigate. *In vitro,* RA has been shown to have a chemotactic effect on neurite outgrowth whereby neurites from chick embryo spinal cord neurons turn and grow up a gradient of RA in typical chemotactic fashion (Maden *et al.,* 1998a). *In vivo,* a similar navigational response can be revealed as it has been demonstrated that RA can alter the projection pathways of sympathetic preganglionic neurons in the chick embryo (Forehand *et al.,* 1998). The axons of the sympathetic neurons enter the spinal cord and turn either rostrally or caudally. At the T2 level they mostly turn rostrally, but at the T6 level they mostly turn caudally. When a bead

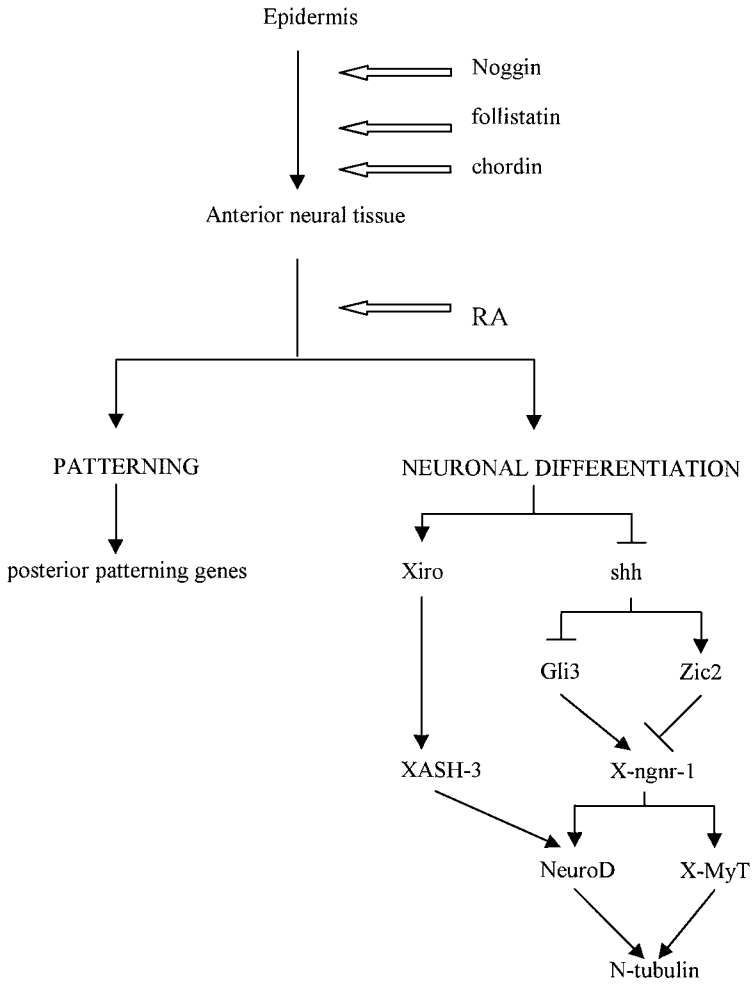



FIG. 4 Summary of how RA acts on the nervous system to generate both anteroposterior pattening (left side) and neuronal differentiation (right side). The neuronal differentiation pathway is taken from Franco *et al.* (1999) and Gomez-Skarmeta *et al.* (1998).

soaked in RA is placed on the T2 somite next to the neural tube, the number of rostrally projecting neurons declines. Conversely, treatment of the T6 level somite with citral or disulphiram increased the number of rostrally projecting neurons. Thus within the thoracic section of the spinal cord there could be a gradient of RA with a high point at the more posterior levels that is responsible for organizing the projection pathways of these preganglionic sympathetic axons.

## 8. RA and *Hox* Genes

The RA effects on neural patterning described above (Sections IV.B.1–5) have led to a deeper understanding of the relationship between RA and *Hox* genes and the role these genes play in establishing anteroposterior pattern. It was originally shown that RA induced *Hox* gene expression in EC cells in a very controlled and precise manner. They are activated in a dose-dependent, 3′–5′ sequential pattern similar to their sequence and time of activation in normal embryonic development (Simeone *et al.,* 1990, 1991) and *in vivo* in response to RA the 3′ genes behave coordinately by spreading in an anterior direction (see Section IV.B.3).

The causal link between the rapid up-regulation of *Hox* genes (within 1 hr of RA treatment) and the aberrant CNS phenotype has been demonstrated by showing that the overexpression of a single *Hox* gene can mimic the RA phenotype. Thus after RA treatment both *Hoxa-1* and *Hoxb-1* are induced to form two stripes of gene expression (in rhombomeres 2 and 4) rather than the normal one (in rhombomere 4) (Alexandre *et al.,* 1996; Conlon and Rossant, 1992). Ectopic expression of *Hoxa-1* either by injecting the mRNA into zebrafish eggs (Alexandre *et al.,* 1996) or by creating transgenic mouse embryos (Zhang *et al.,* 1994) results in the same patterns of ectopic *Hoxb-1* that is caused by RA. Ectopic *Hoxa-2* expression and the same down-regulation of the anterior *Krox-20* stripe in rhombomere 3 also occur. Furthermore, the effect on the anatomy of the zebrafish embryo is exactly the same after ectopic *Hoxa-1* expression or RA treatment: duplicated Mauthner neurons; abnormal development of the jaw apparatus (Meckel's cartilage); fusion of the two streams of neural crest from rhombomere 2 and rhombomere 4 into one stream; and fusion of the trigeminal and facial ganglia (Alexandre *et al.,* 1996). A remarkably similar result is seen with *Hoxb-2;* ectopic expression of *b-2* in zebrafish embryos has the same phenotypic result as RA treatment (Yan *et al.,* 1998).

These experiments clearly show that not only does RA regulate *Hox* genes, but *Hox* genes also regulate each other. But how does RA perform this regulation? There are four clusters of *Hox* genes in the cells of higher vertebrates and in order to explain this coordinated 3′–5′ regulation both *in vivo* and *in vitro* it was suggested that there could be a master regulatory switch operated by RA at the 3′ end of each cluster (Langston and Gudas, 1994). However, the search for RAREs in the enhancer sequences of *Hox* genes has resulted in the discovery of five of them and presumably there will be more. These are in *Hoxa-1* (Frasch *et al.,* 1995; Langston and Gudas, 1992), *Hoxb-1* (Marshall *et al.,* 1994; Studer *et al.,* 1994), *Hoxa-4* (Packer *et al.,* 1998), *Hoxd-4* (Popperl and Featherstone, 1993), and *Hoxb-4* (Gould *et al.,* 1998). So they are certainly not located only at the 3′ end of each cluster, but are also found within clusters and thus subject to individual regulation.

Although *Hox* genes are expressed in the embryo both in the mesoderm and the neurectoderm, these RAREs which have been identified specifically drive *Hox* expression in the neurectoderm, and other enhancers are responsible for mesodermal expression. Thus, for example, point mutations in the *Hoxb-1* RARE

abolishes neurectodermal expression, but not expression in the somites or notochord (Marshall *et al.,* 1994). The same applies to the *Hoxa-1* enhancer (Frasch *et al.,* 1995) and the *d-4* enhancer (Gould *et al.,* 1998; Morrison *et al.,* 1996). These RAREs are not all the same, however, and are of different types, a DR2 (direct repeat with a 2 base pair space in between) in *Hoxb-1* and a DR5 (direct repeat with a 5 base pair space in between) in *Hoxb-4*. Amazingly, when the *b-4* RARE was substituted for the *b-1* RARE, expression of the *b-4* reporter gene was expanded corresponding to approximately the normal boundary of *b-1* expression (Gould *et al.,* 1998). Therefore the sequence composition of the RAREs encodes positional information for positioning the anterior boundaries of *b-1* and *b-4*.

This loss of early expression of *Hox* genes in the neurectoderm caused by mutations in the RAREs is not the end of the story, however, because the later phase of expression of the genes is normal, at least for *b-1* (Studer *et al.,* 1998). This is because there are autoregulatory loops within the *a-1* and *b-1* genes as well as a cross-regulatory loop with *a-1* enhancing *b-1* to establish full expression of *b-1* in rhombomere 4 (Studer *et al.,* 1998). In fact, the early expression of *a-1* is not completely abolished by mutation of its RARE, it is only decreased in level (Dupe *et al.,* 1997) so this is likely to be how the later phase of expression can exist in the absence of the early phase. This explains why the result of mutating the RARE is not as dramatic as a knockout of the same gene, at least for *Hoxa-1* (Dupe *et al.,* 1997). There may, in fact, be other cross-regulatory interactions because after mutation of the *a-1* RARE, *Hoxa-2* levels are lower than normal whereas *Hoxb-2, b-3,* and *a-3* were unaffected (Dupe *et al.,* 1997). Furthermore, in the *Hoxb-1* gene there is also a RARE that operates a repressor of *b-1* in adjacent rhombomers (rhombomeres 3 and 5) so that *b-1* will only be expressed in rhombomere 4 (Studer *et al.,* 1994).

Clearly then the regulation of the *Hox* genes by RA is complex, involving positive enhancers, negative enhancers, and autoregulatory and cross-regulatory interactions. But there is no doubt that endogenous RA, acting via specific types of RARE is instrumental in the establishment of the initial phase of *Hox* gene expression in the neuroectoderm.

## 9. Other Effects of RA on the CNS

Several other later events of RA has on the CNS are of note, but do not readily fit into the categories of early CNS patterning or neuronal differentiation and pathfinding.

First, RA treatment of the chick embryo at tail bud stages leads to caudal agenesis, but interestingly, the truncated end of the embryo contains multiple ectopic neural tube-like structures (Griffith and Wiley, 1991). The same phenomenon occurs in the tail bud of the mouse embryo (Maden and Horton, unpublished, 2000) and so-called neural diverticula, which are seen all along the RA-treated neural tube, may also be a reflection of the same phenonemon (Pauken *et al.,* 1999). The expression of neural genes such as *Pax3* and *Pax6* suggests that these neural

structures in the tail bud are patterened neural tubes (Shum *et al.,* 1999). Neurofilament positive neurons subsequently grow out from them. This is a transformation of mesenchymal tail bud cells into a neural fate that involves the down-regulation of *Wnt-3a* (Shum *et al.,* 1999).

Secondly, mention has been made throughout the above discussion of the effects of RA on the developing eye with regard to the patterning effects—duplication of the eye or the absence of the whole eye or selected parts. But it is also clear that the differentiation of specific cell types within the retina, namely, the photoreceptor cells, is promoted by RA in rat embryos (Kelley *et al.,* 1994) and chick embryos (Stenkamp *et al.,* 1993).

RA also affects another neural structure, the ear, and here it stimulates the regeneration of hair cells. The organ of Corti contains the hair cells and in experiments using neonatal rat pups whose hair cells had been killed with ototoxic drugs, RA induced the regeneration of 78% of the hair cells after 7 days, whereas no hair cells regenerated in controls (Lefebvre *et al.,* 1993). Even in the normal, undamaged organ of Corti RA increased the number of hair cells, resulting in large regions of supernumerary hair cells (Kelley *et al.,* 1993). That this phenomenon is receptor mediated is suggested by the observation that the expression of RAR$\alpha$, RAR$\beta$, and RXR$\alpha$ increases as hair cells differentiate during development (Pauken *et al.,* 1999; Raz and Kelley, 1999) and a RAR$\alpha$ antagonist prevents hair cell development, as does the application of citral, a competitive inhibitor of RA synthesis (Raz and Kelley, 1999).

Finally, a fourth system concerns the differentiation of oligodendrocytes, which are cells that myelinate CNS axons. In contrast to the hair cells described above, RA inhibits the differentiation of oligodendrocytes from rat embryonic spinal cord *in vitro* (Noll and Miller, 1994). Because the migration of oligodendrocytes away from their place of origin in the developing spinal cord into the white matter is a precisely timed event during CNS development, these authors proposed that one of the functions of the high levels of RA in the spinal cord (see Sections III.A–C) is to inhibit the differentiation of oligodendrocytes so that migration takes place. A rather different effect has been reported by Barres *et al.* (1994) on oligodendrocytes from the rat optic nerve. In this case, RA slowed the cell-cycle time and thus promoted their differentiation, so perhaps there is a difference in mechanism between the spinal cord, where RA is present in abundance and the brain where RA is probably present at far lower levels.

## V. Concluding Remarks

The message that this review seeks to get across is that RA is an endogenous molecule in the developing CNS that is involved in multiple aspects of neural development. These aspects are, first, early patterning where it is critical that

there is *no* RA in the presumptive forebrain and midbrain, an intermediate level of RA across the presumptive hindbrain in the form of a gradient so that precise segmental expression patterns of genes such as *Hox* genes, *Krox-20, Kreisler,* etc., can be established within the rhombomeres, and high levels in the presumptive spinal cord. The second aspect is neuronal differentiation. A good deal of emphasis was placed on studies of EC cells because these have been an extremely valuable paradigm for identifying the downstream target genes that are activated by RA during the process of neuronal differentiation and a series of necessary steps can be identified (Table I). This RA-induced process also operates *in vivo* where RA promotes neuronal differentation and a lack of it inhibits neuronal differentiation and a similar scheme involving the up-regulation of proneural and neural genes can be drawn up (Fig. 4). The third aspect is neuronal patterning, where a subset of motoneurons depend on RA produced by adjacent cells that express RALDH2 and a subset of interneurons depend on RA for their differentiation (Fig. 2). The fourth aspect is neurite outgrowth and guidance where a few experiments have suggested that RA could be involved in guiding patterns of neuronal connections within the spinal cord.

These conclusions are backed up by an examination of the distribution of the binding proteins, receptors, and enzymes involved in the RA transduction pathway and an analysis of the altered CNS patterning that occurs following the disruption or overexpression of these molecules. With regard to the patterning aspect of CNS development, one clear message that comes through here is that the hindbrain is the region that is exquisitely sensitive to an alteration in any of the RA-transducing molecules (Table II).

The role of RA in the developing CNS is therefore clearly of interest to developmental biologists and neurobiologists, but these studies also have a great therapeutic potential. Because RA can be used to differentiate EC cells, neuroblastoma cells, or neural stem cells, they can then be used as transplants for the treatment of ischemic damage or Parkinson's disease or to act as a long-term depot for the slow release of relevant compounds. In years to come we may see RA playing an important role in clinical neurology.

## References

Abramovici, A., Kam, J., Liban, E., and Barishak, R. Y. (1978). Incipient histopathological lesions in citral-induced microphthalmos in chick embryos. *Dev. Neurosci.* **1,** 177–185.

Abu-Abed, S. S., Beckett, B. R., Chiba, H., Chithalen, J. V., Jones, G., Metzger, D., Chambon, P., and Petkovich, M. (1998). Mouse P450RAI (CYP26) expression and retinoic acid-inducible retinoic acid metabolism in F9 cells are regulated by retinoic acid receptor g and retinoid X receptor $\alpha$. *J. Biolog. Chem.* **273,** 2409–2415.

Alexandre, D., Clarke, J. D. W., Oxtoby, E., Yan, Y.-L., Jowett, T., and Holder, N. (1996). Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* **122,** 735–746.

Alles, A. J., and Sulik, K. K. (1990). Retinoic acid-induced spina bifida: Evidence for a pathogenic mechanism. *Development,* **108,** 73–81.

Amatruda, T. T., Sidell, N., Ranyard, J., and Koeffler, H. P. (1985). Retinoic acid treatment of human neuroblastoma cells is associated with decreased N-myc expression. *Biochem. Biophys. Res. Commun.* **126,** 1189–1195.

Anchan, R. M., Drake, D. P., Haines, C. F., Gerwe, E. A., and LaMantia, A.-S. (1997). Disruption of local retinoid-mediated gene expression accompanies abnormal development in the mammalian olfactory pathway. *J. Comp. Neurol.* **379,** 171–184.

Ando, M., Yamauchi, M., Fujita, K., Kakita, M., and Nagata, Y. (1996). Induction of tissue transglutaminase in rat superior cervical sympathetic ganglia following *in vitro* stimulation of retinoic acid. *Neurosci. Res.* **24,** 357–362.

Andrews, P. A. (1984). Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Dev. Biol.* **103,** 285–293.

Andrews, P. W., Gonczol, E., Plotkin, S. A., Dignazio, M., and Oosterhuis, J. W. (1986). Differentiation of TERA-2 human embryonal carcinoma cells into neurons and HCMV permissive cells. Induction by agents other than retinoic acid. *Differentiation* **31,** 119–126.

Ang, H. L., and Duester, G. (1997). Initiation of retinoid signalling in primitive streak mouse embryos: Spatiotemporal expression patterns of receptors and metabolic enzymes for ligand synthesis. *Dev. Dynam.* **208,** 536–543.

Ang, H. L., Deltour, L., Hayamizu, T. F., Zgombic-Knight, M., and Duester, G. (1996). Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. *J. Biolog. Chem.* **271,** 9526–9534.

Ang, S.-L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L., and Rossant, J. (1996). A targeted mouse OTX2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122,** 243–252.

Arcangeli, A., Rosati, B., Cherubini, A., Crociani, O., Fontana, L., Passani, B., Wnake, E., and Olivotto, M. (1998). Long term exposure to retinoic acid induces the expression of IRK1 channels in HERG channel-endowed neuroblastoma cells. *Biochem. Biophys. Res. Commun.* **244,** 706–711.

Avantaggiato, V., Acampora, D., Tuorto, F., and Simeone, A. (1996). Retinoic acid induces stage-specific repatterning of the rostral central nervous system. *Dev. Biol.* **175,** 347–357.

Bain, G., Kitchens, D., Yao, M., Huettner, J. E., and Gottlieb, D. I. (1995). Embryonic stem cells express neuronal properties *in vitro*. *Dev. Biol.* **168,** 342–357.

Bain, G., Ray, W. J., Yao, M., and Gottlieb, D. I. (1996). Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem. Biophys. Res. Commun.* **223,** 691–694.

Balkan, W., Colbert, M., Bock, C., and Linney, E. (1992). Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl. Acad. Sci. USA* **89,** 3347–3351.

Bally-Cuif, L., Gulisano, M., Broccoli, V., and Boncinelli, E. (1995). c-otx2 is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryos. *Mech. Dev.* **49,** 49–63.

Bani-Yaghoub, M., Bechberger, J. F., and Naus, C. C. G. (1997). Reduction of connexin 43 expression and dye-coupling during neuronal differentiation of human NTera2/clone D1 cells. *J. Neurosci. Res.* **49,** 19–31.

Bani-Yaghoub, M., Bechberger, J. F., Underhill, T. M., and Naus, C. C. G. (1999). The effects of gap junctional blockage on neuronal differentiation of human NTera2/clone D1 cells. *Exp. Neurol.* **156,** 16–32.

Baroffio, A., Dupin, E., and LeDouarin, N. M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc. Natl. Acad. Sci. USA* **85,** 5325–5329.

Baroffio, A., Dupin, E., and Le Douarin, N. M. (1991). Common precursors for neural crest and mesectodermal derivatives in the cephalic neural crest. *Development* **112,** 301–305.

Barres, B. A., Lazar, M. A., and Raff, M. C. (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development* **120,** 1097–1108.

Bauer, U.-M., Schneider-Hirsch, S., Reinhardt, S., Pauly, T., Maus, A., Wang, F., Heiermann, R., Rentrop, M., and Maelicke, A. (1997). Neuronal cell nuclear factor. A nuclear receptor possibly involved in the control of neurogenesis and neuronal differentiation. *Eur. J. Biochem.* **239,** 826–837.

Bavik, C., Ward, S. J., and Chambon, P. (1996). Developmental abnormalities in cultured mouse embryos deprived of retinoic acid by inhibition of yolk-sac retinol binding protein synthesis. *Proc. Natl. Acad. Sci. USA* **93,** 3110–3114.

Beczkowska, I. W., Grazy, K. N., Pickel, V. M., and Inturrisi, C. E. (1997). Inducible expression of *N*-methyl-D-aspartate receptor and delta and mu opiod receptor messenger RNAs and protein in the NT2-N human cell line. *Neuroscience* **79,** 855–862.

Belliveau, D. J., Bechberger, J. F., Rogers, K. A., and Naus, C. C. (1997). Differential expression of gap junctions in neurons and astrocytes derived from P19 embryonal carcinoma cells. *Dev. Genet.* **21,** 187–200.

Berger, C., Reinhardt, S., Rentrop, M., Bachmann, M., Weiser, T., Link, E., Wienrich, M., Jahn, R., and Maelicke, A. (1997). *De novo* acquisition of neuronal polarity in retinoic acid-treated embryonal carcinoma cells. *Eur. J. Cell Biol.* **74,** 230–245.

Berggren, K., McCaffery, P., Drager, U., and Forehand, C. J. (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. *Dev. Biol.* **210,** 288–304.

Berrard, S., Faucon Biguet, N., Houhou, L., Lamouroux, A., and Mallet, J. (1993). Retinoic acid induces cholinergic differentiation of cultured newborn rat sympathetic neurons. *J. Neurosci. Res.* **35,** 382–389.

Black, B. L., Ligon, K. L., Zhang, Y., and Olson, E. N. (1996). Cooperative transcriptional activation by the neurogenic basic helix–loop–helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. *J. Biolog. Chem.* **271,** 26659–26663.

Blaner, W. S., Piantedosi, R., Sykes, A., and Vogel, S. (1999). Retinoic acid synthesis and metabolism. *In* "Retinoids: The biochemical and molecular basis of vitamin A and retinoid action" (H. Nau and W. S. Blaner, Eds.), pp. 117–149. Springer-Verlag, Berlin.

Blumberg, B., Mangelsdorf, D. J., Dyck, J. A., Bittner, D. A., Evans, R. M., and De Robertis, E. M. (1992). Multiple retinoid-responsive receptors in a single cell: Families of retinoid "X" receptors and retinoic acid receptors in the *Xenopus* egg. *Proc. Natl. Acad. Sci. USA* **89,** 2321–2325.

Blumberg, B., Bolado, J., Moreno, T. A., Kintner, C., Evans, R. M., and Papalopulu, N. (1997). An essential role for retinoid signalling in anteroposterior neural patterning. *Development* **124,** 373–379.

Borlongan, C. V., Tajima, A., Trojanowski, J. Q., Lee, V. M. Y., and Sanberg, P. R. (1998). Transplantation of cryopreserved human embryonal carcinoma derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp. Neurol.* **149,** 310–321.

Boudjelal, M., Taneja, R., Matsubara, S., Bouillet, P., Dolle, P., and Chambon, P. (1997). Overexpression of Stra 13, a novel retinoic acid-inducible gene of the basic helix–loop–helix family, inhibits mesoderm and promotes neuronal differentiation of P19 cells. *Genes Dev.* **11,** 2052–2065.

Bouillet, P., Oulad-Abdelghani, M., Vicaire, S., Garnier, J.-M., Schuhbaur, B., Dolle, P., and Chambon, P. (1995a). Efficient cloning of cDNAs of retinoic acid- responsive genes in P19 embryonal carcinoma cells and characterisation of a novel mouse gene, Stra 1 (mouse LERK-2/Eplg2). *Dev. Biol.* **170,** 420–433.

Bouillet, P., Chazaud, C., Oulad-Abdelghani, M., Dolle, P., and Chambon, P. (1995b). Sequence and expression pattern of the Stra2 (Gbx-2) homoebox-containing gene induced by retinoic acid in P19 embryonal carcinoma cells. *Dev. Dynam.* **204,** 372–382.

Bunone, G., Borrello, M. G., Picetsi, R., Bongarzone, I., Peverali, F. A., De Franciscis, V., Della Valle, G., and Pierotti, M. A. (1995). Induction of RET proto-oncogene expression in neuroblastoma cells precedes neuronal differentiation and is not mediated by protein synthesis. *Exp. Cell Res.* **217,** 92–99.

Bussiere, M., Campenot, R. B., Ure, D. R., Vance, J. E., and Vance, D. E. (1995). Reciprocal regulation

of choline acetyltransferase and choline kinase in sympathetic neurons during cholinergic differentiation. *Biochem. Biophys. Acta* **1259,** 148–154.

Casper, D., and Davies, P. (1989). Stimulation of choline acetyltransferase activity by retinoic acid and sodium butyrate in a cultured human neuroblastoma. *Brain Res.* **478,** 74–84.

Castle, V. P., Ou, X., O'Shea, S., and Dixit, V. M. (1992). Induction of thrombospondin 1 by retinoic acid is important during differentiation of neuroblastoma cells. *J. Clin. Invest.* **90,** 1857–1863.

Castro-Obregon, S., and Covarrubias, L. (1996). Role of retinoic acid and oxidative stress in embryonic stem cell death and neuronal differentiation. *FEBS Lett.* **381,** 93–97.

Cauley, K., Marks, M., Gahring, L. C., and Rogers, S. W. (1996). Nicotinic receptor subunits a3, a4, and b2 and hign affinity nicotine binding sites are expressed by P19 embryonal cells. *J. Neurobiol.* **30,** 303–314.

Chambout-Guerin, A. M., Martinez, M. C., Hamimi, C., Gauthereau, X., and Nunez, J. (1995). Tumor necrosis factor receptors in neuroblastoma SKNBE cells and their regulation by retinoic acid. *J. Neurochem.* **65,** 537–544.

Chambaut-Guerin, A. M., Herigault, S., Rouet-Benzineb, P., Rouher, C., and Lafuma, C. (2000). Induction of matrix metalloproteinase MMP-9 (92-kDa gelatinase) by retinoic acid in human neuroblastoma SKNBE cells: Relevance to neuronal differentiation. *J. Neurochem.* **74,** 508–517.

Chandrasekaran, V., Zhai, Y., Wagner, M., Kaplan, P. L., Napoli, J. L., and Higgins, D. (2000). Retinoic acid regulates the morphological development of sympathetic neurons. *J. Neurobiol.* **42,** 383–393.

Chen, J., and Kelley, P. T. (1996). Retinoic acid stimulates a-CAMKII gene expression in PC12 cells at a distinct transcription initiation site. *J. Neurosci.* **16,** 5704–5714.

Chen, W.-H., Morriss-Kay, G. M., and Copp, A. J. (1995). Genesis and prevention of spinal neural tube defects in the curly tail mutant mouse: Involvement of retinoic acid and its nuclear receptors RAR-B and RAR-g. *Development* **121,** 681–691.

Cheung, W. M. W., Chu, A. H., and Ip, N. Y. (1997). Identification of candidate genes induced by retinoic acid in embryonal carcioma cells. *J. Neurochem.* **68,** 1882–1888.

Chiang, M. Y., Misner, D., Kempermann, G., Schikorski, T., Giguere, V., Sucov, H. M., Gage, F. H., Stevens, C. F., and Evans, R. M. (1998). An essential role for retinoid receptors RARbeta and RXRgamma in long-term potentiation and depression. *Neuron* **21,** 1353–1361.

Cho, K. W. Y., and De Robertis, E. M. (1990). Differential activation of *Xenopus* homeobox genes by mesoderm-inducing growth factors and retinoic acid. *Genes Dev.* **4,** 1910–1916.

Cockshut, A. M., Jonet, L., Jeanny, J.-C., Vigny, M., and Raulais, D. (1994). Retinoic acid induced heparin-binding protein expression and localization during gastrulation, neurulation, and organogenesis. *Dev. Dynam.* **200,** 198–211.

Cohlan, S. Q. (1953). Excessive intake of vitamin A as a cause of congenital abnormalities in the rat. *Science* **117,** 535–536.

Colbert, M. C., Linney, E., and LaMantia, A. S. (1993). Local sources of retinoic acid coincide with retinoid-mediated transgene activity during embryonic development. *Proc. Natl. Acad. Sci. USA* **90,** 6572–6576.

Colbert, M. C., Rubin, W. W., Linney, E., and LaMantia, A. S. (1995). Retinoid signalling and the generation of regional and celluar diversity in the embryonic mouse spinal cord. *Dev. Dynam.* **204,** 1–12.

Coleman, B. A., and Taylor, P. (1996). Regulation of acetylcholinesterase expression during neuronal differentiation. *J. Biolog. Chem.* **271,** 4410–4416.

Conlon, R. A., and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes *in vivo*. *Development* **116,** 357–368.

Connor, M. J., and Smit, M. H. (1987). Terminal-group oxidation of retinol by mouse epidermis. *Biochem. J.* **244,** 489–492.

Corcoran, J., and Maden, M. (1999). Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nature Neurosci.* **2,** 307–308.

Corcoran, J., Shroot, B., Pizzey, J., and Maden, M. (2000). The role of retinoic acid receptors in neurite

outgrowth from different populations of embryonic mouse dorsal root ganglia. *J. Cell Sci.* **113,** 2567–2574.

Cosgaya, J. M., Garcia-Villalba, P., Perena, R., and Aranda, A. (1996). Comparison of the effects of retinoic acid and nerve growth factor on PC12 cell proliferation, differentiation and gene expression. *J. Neurochem.* **66,** 89–98.

Cosgaya, J. M., Perone, R., and Aranda, A. (1997). Retinoic acid induces secretion of transforming growth factors by PC12 pheochromocytoma cells. *Oncogene* **14,** 579–587.

Cullenor, J. G., Evin, G., Cooney, M. A., Wardan, H., Sharples, R. A., Maher, F., Reed, G., Diehlmann, A., Weidemann, A., Beyreuther, K., and Masters, C. L. (2000). Presenilin 2 expression in neural cells: Induction during differentiation of embyronal carcinoma cells. *Exp. Cell Res.* **255,** 192–206.

Cunningham, M. L., MacAuley, A., and Mirkes, P. E. (1994). From gastrulation to neurulation: Transition in retinoic acid sensitivity identifies distinct stages of neural patterning in the rat. *Dev. Dynam.* **200,** 227–241.

Dekker, E.-J., Pannese, M., Houtzager, E., Boncinelli, E., and Durston, A. (1992). Colinearity in the *Xenopus laevis Hox-2* complex. *Mech. Dev.* **40,** 3–12.

Dekker, E.-J., Vaessen, M.-J., van den Berg, C., Timmermans, A., Godsave, S., Holling, T., Niewkoop, P., van Kessel, A. G., and Durston, A. (1994). Overexperssion of a cellular retinoic acid binding protein (xCRABP) causes anteroposterior defects in developing *Xenopus* embryos. *Development* **120,** 973–985.

de la Monte, S. M., Xu, Y. Y., and Wands, J. R. (1996). Modulation of neuronal thread protein expression with neuritic sprouting: Relevance to Alzheimer's diesase. *J. Neurol. Sci.* **138,** 26–35.

Deltour, L., Foglio, M. H., and Duester, G. (1999). Impaired retinol utilisation in Adh4 alcohol dehydrogenase mutant mice. *Dev. Genet.* **25,** 1–10.

Dencker, L., Annerrwall, E., Busch, C., and Eriksson, U. (1990). Localization of specific retinoid-binding sites and expression of cellular retinoic acid-binding protein (CRABP) in the early mouse embryo. *Development* **110,** 343–352.

de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J., and van der Saag, P. T. (1999). Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and *Xenopus laevis* embryogenesis. *Mech. Dev.* **82,** 205–211.

Dersch, H., and Zile, M. H. (1993). Induction of normal cardiovascular development in the vitamin A-deproved quail embryo by natural retinoids. *Dev. Biol.* **160,** 424–433.

Deschamps, J., de Laaf, R., Joosen, L., Meijlink, F., and Destree, O. (1987a). Abundant expression of homeobox genes in mouse embryonal carcinoma cells correlates with chemically induced differentiation. *Proc. Natl. Acad. Sci. USA* **84,** 1304–1308.

Deschamps, J., de Laaf, R., Verrijzer, P., de Gouw, M., Destree, O., and Meijlink, F. (1987b). The mouse *Hox2.3* homeobox-containing gene: Regulation in differentiating pluripotent stem cells and expression pattern in embryos. *Differentiation* **35,** 21–30.

Dickman, E. D., Thaller, C., and Smith, S. M. (1997). Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* **124,** 3111–3121.

Die-Smulders, C. E. M., Sturkenboom, M. C. J. M., Veraart, J., van Katwijk, C., Sastrowijoto, P., and van der Linden, E. (1995). Severe limb defects and craniofacial anomalies in a fetus conceived during acitretin therapy. *Teratology* **52,** 215–219.

Dimitroulakos, J., Pienkowska, M., Sun, P., Farooq, S., Zielenska, M., Squire, J. A., and Yegler, H. (1999). Identification of a novel zinc finger gene zf5-3 as a potential mediator of neuroblastoma differentiation. *Int. J. Cancer* **81,** 970–978.

Dinsmore, J. H., and Solomon, F. (1991). Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell* **64,** 817–826.

Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G., and Chambon, P. (1990). Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110,** 1133–1151.

Dolle, P., Fraulob, V., Kastner, P., and Chambon, P. (1994). Developmental expression of murine retinoid X receptor (RXR) genes. *Mech. Dev.* **45,** 91–104.

Dong, D., and Zile, M. H. (1995). Endogenous retinoids in the early avian embryo. *Biochem. Biophys. Res. Commun.* **217,** 1026–1031.

Duester, G. (1996). Involvement of alcohol dehydrogenase, short-chain dehydrogenase/reductase, aldehyde dehydrogenase, and cytochrome P450 in the control of retinoid signalling by activation of retinoic acid synthesis. *Biochemistry* **35,** 12221–12227.

Dunlop, J., McIlvain, H. B., Lou, Z., and Franco, R. (1998). The pharmacological profile of L-glutamate transport in human NT2 neurones is consistent with excitatory amino acid transporter 2. *Eur. J. Pharmacol.* **360,** 249–256.

Dupe, V., Davenne, M., Brocard, J., Dolle, P., Mark, M., Dierich, A., Chambon, P., and Rijli, F. M. (1997). *In vivo* functional analysis of the *Hoxa-1* 3′ retinoic acid response element (3′RARE). *Development* **124,** 399–410.

Dupe, V., Ghyselinck, N., Wendling, O., Chambon, P., and Mark, M. (1999). Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. *Development* **126,** 5051–5059.

Dupin, E., and Le Douarin, N. M. (1995). Retinoic acid promotes the differentiation of adrenergic cells and melanocytes in quail neural crest cultures. *Dev. Biol.* **168,** 529–548.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendricks, H. F. J., de Vries, N. J., Heideveld, M., and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing nervous system. *Nature (Lond.)* **340,** 140–144.

Edwards, M. K. S., and McBurney, M. W. (1983). The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev. Biol.* **98,** 187–191.

Edwards, R. B., Adler, A. J., Dev, S., and Claycomb, R. C. (1992). Synthesis of retinoic acid from retinol by cultured rabbit Muller cells. *Exp. Eye Res.* **54,** 481–490.

Ehrhard, P. B., Ganter, U., Schmutz, B., Bauer, J., and Otten, U. (1993). Expression of low-affinity NGF receptor and TrkB mRNA in human SH-SY5Y neuroblastoma cells. *FEBS Lett.* **330,** 287–292.

Encinas, M., Iglesias, M., Llecha, N., and Comella, J. X. (1999). Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *J. Neurochem.* **73,** 1409–1421.

Ensini, M., Tsuchida, T., Betling, H-G., and Jessell, T. (1998). The control of rostrocaudal pattern in the developing spinal cord: Specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm. *Development* **125,** 969–982.

Enwright, J. F., and Grainger, R. M. (2000). Altered retinoid signaling in the heads of small eye mouse embryos. *Dev. Biol.* **221,** 10–22.

Esdar, C., Oehrlein, S. A., Reinhardt, S., Maelicke, A., and Herget, T. (1999). The protein kinase C (PKC) substrate GAP-43 is already expressed in neuronal precursor cells, localises with PKCn and binds calmodulin. *Eur. J. Neurosci.* **11,** 503–516.

Fan, F. S., Shen, H.-H., Tseng, W.-P., Chen, P.-O., and Tsai, S.-F. (1998). Molecular cloning and characterisation of a human brain-specific gene implicated in neuronal differentiation. *Mol. Brain Res.* **54,** 113–123.

Faraonio, R., Moffatt, P., LaRochelle, O., Schipper, H. M., S-Arnaud, R., and Seguin, C. (2000). Characterisation of *cis*-acting elements in the promoter of the mouse metallothionein-3 gene. Activation of gene expression during neuronal differentiation of P19 embryonal carcinoma cells. *Eur. J. Biochem.* **267,** 1743–1753.

Farooqui, S. M. (1994). Induction of adenyl cyclase sensitive dopamine D2-receptors in retinoic acid induced differentiated human neuroblastoma SHSY-5Y cells. *Life Sciences* **55,** 1887–1893.

Fawcett, D., Pasceri, P., Fraser, R., Colbert, M., Rossant, J., and Giguere, V. (1995). Postaxial polydactyly in forelimbs of CRABP-II mutant mice. *Development* **121,** 671–679.

Feng, Z., and Porter, A. G. (1999). NF-kB/rel proteins are required for neuronal differentiation of SH-SY5Y neuroblastoma cells. *J. Biolog. Chem.* **274,** 30341–30344.

Ferrari, N., Pfahl, M., and Levi, G. (1998). Retinoic acid receptor $\gamma 1$ (RAR$\gamma 1$) levels control RAR$\beta 2$ expression in SK-N-BE2(c) neuroblastoma cells and regulate a differentiation–apoptosis switch. *Mol. Cell Biol.* **18,** 6482–6492.

Finley, M. F. A., Kulkarni, N., and Huettner, J. E. (1996). Synapse formation and establishment of neuronal polarity by P19 embryonic carcinoma cells. *J. Neurosci.* **16,** 1056–1065.

Fischer, R., Shea, T. B., Sapirstein, V. S., and Kosik, K. S. (1985). Expression and distribution of microtubule-associated protein 2 (MAP2) in neuroblastoma and primary neuronal cells. *Devl. Brain Res.* **25,** 99–109.

Forehand, C. J., Ezerman, E. B., Goldblatt, J. P., Skidmore, D. L., and Glover, J. C. (1998). Segment-specific pattern of sympathetic preganglionic projections in the chicken embryo spinal cord is altered by retinoids. *Proc. Natl. Acad. Sci. USA* **95,** 10878–10883.

Fraichard, A., Chassandre, O., Bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995). *In vitro* differentiation of embryonic stem cells into glial cells and functional neurons. *J. Cell Sci.* **108,** 3181–3188.

Franco, P. G., Paganelli, A. R., Lopez, S. L., and Carrasco, A. E. (1999). Functional association of retinoic acid and hedgehog signaling in *Xenopus* primary neurogenesis. *Development* **126,** 4257–4265.

Frasch, M., Chen, X., and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa-1* and *Hoxa-2* loci in both mice and *Drosophila*. *Development* **121,** 957–974.

Fujii, H., and Hamada, H. (1993). A CNS-specific POU transcription factor, Brn-2, is required for establishing mammalian neural cell lineages. *Neuron* **11,** 1197–1206.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S., and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16,** 4163–4173.

Fukuhara, S., Mukai, H., and Munekata, E. (1997). Activin A and all-*trans*-retinoic acid cooperatively enhanced the functional activity of L-type $Ca^{2+}$ channels in the neuroblastoma C1300 cell line. *Biochem. Biophys. Res. Commun.* **241,** 363–368.

Gaetano, C., Matsuo, T., and Thiele, C. J. (1997). Identification and characterisation of a retinoic acid-regulated human homologue of the unc-33-like phosphoprotein gene (hUlip) from neuroblastoma cells. *J. Biolog. Chem.* **272,** 12195–12201.

Gajovic, S., St.-Onge, L., Yokota, Y., and Gruss, P. (1997). Retinoic acid mediates Pax6 expression during *in vitro* differentiation of embryonic stem cells. *Differentiation* **62,** 187–192.

Gale, E., Prince, V., Lumsden, A., Clarke, J., Holder, N., and Maden, M. (1996). Late effects of retinoic acid on neural crest and aspects of rhombomere. *Development* **122,** 783–793.

Gale, E., Zile, M., and Maden, M. (1999). Hindbrain respecification in the retinoid-deficient quail. *Mech. Dev.* **89,** 43–54.

Gao, Z., Chen, M., Collins, H. W., Matschinsky, F. M., Lee, V. M. Y., and Wolf, B. A. (1998a). Mechanisms of spontaneous cytosolic $Ca^{2+}$ transients in differentiated human neuronal cells. *Eur. J. Neurosci.* **10,** 2416–2425.

Gao, Z., Xu, G., Stwora-Wojczyk, M-M., Matschinsky, F. M., Lee, V. M. Y., and Wolf, B. A. (1998b). Retinoic acid induction of calcium channel expression in human NT2N neurons. *Biochem. Biophys. Res. Commun.* **247,** 407–413.

Garcia, M. A., Vasquez, J., Gomenez, C., Valdivieso, F., and Zafra, F. (1996). Transcription factor AP-2 regulates human apolipoprotein E gene expression in astrocytoma cells. *J. Neurosci.* **16,** 7550–7556.

Garel, S., Marin, F., Grosschedl, R., and Charnay, P. (1999). Ebf1 controls early cell differentiation in the embryonic striatum. *Development* **126,** 5285–5294.

Ghyselinck, N. B., Dupe, V., Dierich, A., Massaddeq, N., Garnier, J.-M., Rochette-Egly, C., Chambon, P., and Mark, M. (1997). Role of the retinoic acid receptor beta (RAR$\beta$) during mouse development. *Int. J. Dev. Biol.* **41,** 425–447.

Ghyselinck, N. B., Bavik, C., Sapin, V., Mark, M., Bonnier, D., Hindelang, C., Dierich, A., Nilsson, C. B., Hakansson, H., Sauvant, P., Azias-Braesco, V., Frasson, M., Picaud, S., and Chambon, P. (1999). Cellular retinol-binding protein I is essential for vitamin A homeostasis. *EMBO J.* **18,** 4903–4914.

Gill, R. M., Slack, R., Kless, M., and Hamel, P. A. (1998). Regulation of expression and activity of distinct pRB, E2F, D-type cyclin and CK1 family members during terminal differentiation of P19 cells. *Exp. Cell Res.* **244,** 157–170.

Godbout, R., Packer, M., Poppema, S., and Dabbath, L. (1996). Localization of cytosolic aldehyde dehydrogenase in the developing chick retina: *In situ* hydridisation and immunohistochemical analyses. *Dev. Dynam.* **205,** 319–331.

Goddard, J. M., Rossel, M., Manley, N. R., and Capecchi, M. R. (1996). Mice with targeted disruption of *Hoxb-1* fail to form the motor nucleus of the VIIth nerve. *Development* **122,** 3217–3228.

Godsave, S. F., Koster, C. H., Getahun, A., Mathu, M., Hooiveld, M., van der Wees, J., Hendrick, J., and Durston, A. J. (1998). Graded retinoid responses in the developing hindbrain. *Dev. Dynam.* **213,** 39–49.

Gomez-Skarmeta, J., Glavic, A., de la Calle- Mustienes, E., Modolell, J., and Mayor, R. (1998). *Xiro,* a *Xenopus* homolog of the *Drosophila Iroquois* complex genes, control development at the neural plate. *EMBO J.* **17,** 181–190.

Gorry, P., Lufkin, T., Dierich, A., Rochette-Egly, C., Decimo, D., Dolle, P., Mark, M., Durand, B., and Chambon, P. (1994). The cellular retinoic acid binding protein I is dispensible. *Proc. Natl. Acad. Sci. USA* **91,** 9032–9036.

Gould, A., Itasaki, N., and Krumlauf, R. (1998). Initiation of rhombomeric *Hoxb4* expression requires induction by somites and a retinoid pathway. *Neuron* **21,** 39–51.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10,** 1135–1147.

Grapin-Botton, A., Bonnin, M. A., and Le Douarin, N. M. (1997). *Hox* gene induction in the neural tube depends on three parameters: Competence, signal supply and paralogue group. *Development* **124,** 849–859.

Grapin-Botton, A., Bonnin, M.-A., Sieweke, M., and Le Douarin, N. M. (1998). Defined concentrations of a posteriorizing signal are critical for MafB/Kreisler segmental expression in the hindbrain. *Development* **125,** 1173–1181.

Griffith, C. M., and Wiley, M. J. (1991). Effects of retinoic acid on chick tail bud development. *Teratology* **43,** 217–224.

Grondona, J. M., Kastner, P., Gansmuller, A., Decimo, D., Chambon, P., and Mark, M. (1996). Retinal dysplasia and degeneration in RAR$\beta$2/RAR$\gamma$2 compound mutant mice. *Development* **122,** 2173–2188.

Gustafson, A.-L., Eriksson, U., and Dencker, L. (1999). CRBPI and CRABPI localisation during olfactory nerve development. *Dev. Brain Res.* **114,** 121–126.

Hale, F. (1933). Pigs born without eye balls. *J. Hered.* **24,** 105–106.

Haselbeck, R. J., Hoffmann, I., and Duester, G. (1999). Distinct functions for Aldh1 and Raldh2 in the control of ligand production for embryonic retinoid signalling pathways. *Dev. Genet.* **25,** 353–364.

Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y., Shibai, H., and Muramatsu, M. (1990). Activin/EDF as an inhibitor of neural differentiation. *Biochem. Biophys. Res. Commun.* **173,** 193–200.

Hashino, E., Johnson, E. M., Milbrandt, J., Shero, M., Salvi, R. J., and Cohan, C. S. (1999). Multiple actions of neurutin correlate with spatiotemporal patterns of ret expression in developing chick cranial ganglion neurons. *J. Neurosci.* **19,** 8476–8486.

Haskell, B. E., Stach, R. W., Werrbach-Perez, K., and Perez-Polo, J. R. (1987). Effect of retinoic acid in nerve growth factor receptors. *Cell Tissue Res.* **247,** 67–73.

Heck, S., Enz, R., Richter-Landsberg, C., and Blohm, D. H. (1997a). Expression and mRNA splicing of glycine receptor subunits and gephyrin during neuronal differentiation of P19 cells *in vitro*: Studies by RT-PCR and immunocytochemistry. *Dev. Brain Res.* **98,** 211–220.

Heck, S., Enz, R., Richter-Landsberg, C., and Blohm, D. H. (1997b). Expression of eight metabotropic glutamate receptor subtypes during neuronal differentiation of P19 embryonal carcinoma cells: A study by RT-PCR and *in situ* hybridisation. *Dev. Brain Res.* **101,** 85–91.

Heiermann, R., Rentrop, M., Lang, E., and Maelicke, A. (1992). Expression of retinoic acid nuclear receptors in the mouse embryonal carcinoma cell line PCC7-Mz1. *FEBS Lett.* **312,** 75–79.

Heine, U. I., Roberts, A. B., Munoz, E. F., Roche, N. S., and Sporn, M. B. (1985). Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. *Virchow's Arch. B Cell Pathol.* **50,** 135–152.

Hendry, I. A., and Belford, D. A. (1991). Retinoic acid potentiates the neurotrophic but not the mitogenic action of the class 1 heparin-binding growth factor (HBGF-1). *Brain Res.* **542,** 29–34.

Henion, P. D., and Weston, J. A. (1994). Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest populations. *Dev. Biol.* **161,** 243–250.

Hens, J., Nuydens, R., Geerts, H., Senden, N. H. M., van de Ven, W. J. M., Roebroek, A. J. M., van de Velde, H. J. K., Ramaekers, F. C. S., and Broers, J. L. V. (1998). Neuronal differentiation is accompanied by NSP-C expression. *Cell Tissue Res.* **292,** 229–237.

Herget, T., Specht, H., Esdar, C., Oehrlein, S. A., and Maelicke, A. (1998). Retinoic acid induces apoptosis-associated neural differentiation of a murine teratocarcinoma cell line. *J. Neurosci.* **70,** 47–58.

Hernandez, M.-C., Andres-Barquin, P. J., Holt, I., and Israel, M. A. (1998). Cloning of human ENC-1 and evaluation of its expression and regulation in nervous system tumors. *Exp. Cell Res.* **244,** 470–477.

Hill, D. R., and Robertson, K. A. (1997). Characterisation of the colinergic neuronal differentiation of the human neuroblastoma cell line LAN-5 after treatment with retinoic acid. *Dev. Brain Res.* **102,** 53–67.

Hill, J., Clarke, J. D. W., Vargesson, N., Jowett, T., and Holder, N. (1995). Exogenous retinoic acid causes specific alterations in the development of the midbrain and hindbrain of the zebrafish embryo including positional respecification of the Mauthner neuron. *Mech. Dev.* **50,** 3–16.

Ho, L., Mercola, M., and Gudas, L. J. (1994). *Xenopus laevis* cellular retinoic acid-binding protein: Temporal and spatial expression pattern during early embryogenesis. *Mech. Dev.* **47,** 53–64.

Holder, N., and Hill, J. (1991). Retinoic acid modifies development of the midbrain–hindbrain border and affects cranial ganglion formation in zebrafish embryos. *Development* **113,** 1159–1170.

Hollemann, T., Chen, Y., Grunz, H., and Pieler, T. (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17,** 7361–7372.

Honda, S., Itoh, F., Yoshimoto, M., Hinoda, Y., and Imai, K. (1998). Changes in morphology of neuroblastoma cells treated with all-*trans*-retinoic acid combined with transfer of the C-terminal region of the amyloid precursor protein. *J. Clin. Lab. Anal.* **12,** 172–178.

Hong, C. S., Caromile, L., Nomata, Y., Mori, H., Bredesen, D. E., and Koo, E. H. (1999). Contrasting role of presenilin-1 and presenilin-2 in neuronal differentiation *in vitro*. *J. Neurosci.* **19,** 637–643.

Hoover, F., and Glover, J. C. (2000). Regional pattern of retinoid X receptor-$\alpha$ gene expression in the central nervous system of the chicken embryo and its up-regulation by exposure to 9-*cis* retinoic acid. *J. Comp. Neurol.* **398,** 575–586.

Hoover, F., Kielland, A., and Glover, J. C. (2000). RXR$\gamma$ gene is expressed by discrete cell columns within the alar plate of the brainstem of the chicken embryo. *J. Comp. Neurol.* **416,** 417–428.

Horn, V., Minucci, S., Ogryzko, V. V., Adamson, E. D., Howard, B. H., Levin, A. A., and Ozato, K. (1996). RAR and RXR selective ligands cooperatively induce apoptosis and neuronal differentiation in P19 embryonal carcinoma cells. *FASEB J.* **10,** 1071–1077.

Horton, C., and Maden, M. (1995). Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. *Dev. Dynam.* **202,** 312–323.

Huang, S., and Sato, S. (1998). Progenitor cells in the adult zebrafish nervous system express a Brn-1-related POU gene, tai-ji. *Mech. Dev.* **71,** 23–35.

Hung, A. Y., Koo, E. H., Haass, C., and Selkoe, D. J. (1992). Increased expression of $\beta$-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage. *Proc. Natl. Acad. Sci. USA* **89,** 9439–9443.

Hunter, K., Maden, M., Summerbell, D., Eriksson, U., and Holder, N. (1991). Retinoic acid stimulates neurite outgrowth in the amphibian spinal cord. *Proc. Natl. Acad. Sci. USA* **80,** 5525–5529.

Hyatt, G. A., Schmitt, E. A., Marsh-Armstrong, N. R., and Dowling, J. E. (1992). Retinoic acid-induced duplication of the zebrafish retina. *Proc. Natl. Acad. Sci. USA* **89,** 8293–8297.

Ido, A., Miura, Y., and Tamaoki, T. (1994). Activation of ATBF1, a multiple-homeodomain zinc-finger gene, during neuronal differentiation of murine embryonal carcinoma cells. *Dev. Biol.* **163,** 184–187.

Inoue, S., Orimo, A., Saito, T., Ikeda, K., Sakata, K., Hosoi, T., Orimo, H., Ouchi, Y., and Muramatsu, M. (1997). A novel RING finger protein, BFP, predominantly expressed in the brain. *Biochem. Biophys. Res. Commun.* **240,** 8–14.

Inouye, S., Seo, M., Yamada, Y., and Nakazawa, A. (1998). Increase of adenylate kinase isozyme 1 protein during neuronal differentiation in mouse embryonal carcinoma P19 cells and in rat brain primary cultured cells. *J. Neurochem.* **71,** 125–133.

Irving, C., and Mason, I. (2000). Signalling by FGF8 from the isthmus patterns anterior hindbrain and establishes the anterior limit of *Hox* gene expression. *Development* **127,** 177–186.

Itasaki, N., Sharpe, J., Morrison, A., and Krumlauf, R. (1996). Reprogramming Hox expression in the vertebrate hindbrain: Influence of paraxial mesoderm and rhombomere transposition. *Neuron* **16,** 487–500.

Jacob, A., Budhiraja, S., and Reichel, R. R. (1997). Differential induction of HNF-3 transcription factors during neuronal differentiation. *Exp. Cell Res.* **234,** 277–284.

Johnson, J. E., Zimmerman, K., Saito, T., and Anderson, D. J. (1992). Induction and repression of mammalian achaete-scute homologue (MASH) gene expression during neuronal differentiation of P19 embryonal carcinoma cells. *Development* **114,** 75–87.

Jones, B. B., Ohno, C. K., Allenby, G., Boffa, M. B., Levin, A. A., Grippo, J. F., and Petkovich, M. (1995). New retinoid X receptor subtypes in zebra fish (*Danio rerio*) differentially modulate transcription and do not bind 9-*cis* retinoic acid. *Mol. Cell Biol.* **15,** 5226–5234.

Jones-Villeneuve, E. M. V., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94,** 253–262.

Jonk, L. J. C., de Jonge, M. E. J., Vervaart, J. M. A., Wissink, S., and Kruijer, W. (1994). Isolation and developmental expression of retinoic acid-induced genes. *Dev. Biol.* **161,** 604–614.

Joore, J., van der Lans, G. B. L. J., Lanser, P. H., Vervaart, J. M. A., Zivkovic, D., Speksnijder, J. E., and Kruijer, W. (1994). Effects of retinoic acid on the expression of retinoic acid receptors during zebrafish embryogenesis. *Mech. Dev.* **46,** 137–150.

Joore, J., Timmermans, A., van de Water, S., Folkers, G. E., van der Saag, P. T., and Zivkovic, D. (1997). Domains of retinoid signalling and neurectodermal expression of zebrafish *otx1* and *goosecoid* are mutually exclusive. *Biochem. Cell Biol.* **75,** 601–612.

Jostock, R., Rentrop, M., and Maelicke, A. (1998). Cell fate specification in an *in vitro* model of neural development. *Eur. J. Cell Biol.* **76,** 63–76.

Kadomatsu, K., Huang, R.-P., Suganuma, T., Murata, F., and Muramatsu, T. (1990). A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in a spatially and temporally controlled manner during mouse embryogenesis. *J. Cell Biol.* **110,** 607–616.

Kalter, H., and Warkany, J. (1959). Experimental production of congenital malformations in mammals by metabolic procedure. *Physiol. Rev.* **39,** 69–115.

Kaplan, D. R., Matsumoto, K., Lucarelli, E., and Thiele, C. J. (1993). Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation to human neuroblastoma cells. *Neuron* **11,** 321–331.

Kasckow, J. W., Parkes, D. G., Owens, M. J., Stipetic, M. D., Han, J. H., Nemeroff, C. B., and

Vale, W. W. (1994). The BE(2)-M17 neuroblastoma cell line synthesises and secretes corticotropin-releasing factor. *Brain Res.* **654,** 159–162.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J.-L., Dolle, P., and Chambon, P. (1994a). Genetic analysis of RXRa developmental function: Convergence of RXR and RAR signalling pathways in heart and eye morphogenesis. *Cell* **78,** 987–1003.

Kastner, P., Chambon, P., and Leid, M. (1994b). Role of nuclear retinoic acid receptors in the regulation of gene expression. *In* "Vitamin A in health and disease" (R. Blomhoff, Ed.), pp. 189–238. Marcel Dekker Inc., New York.

Kastner, P., Mark, M., and Chambon, P. (1995). Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life?. *Cell* **83,** 859–869.

Kastner, P., Mark, M., Ghyselinck, N., Kretzel, W., Dupe, V., and Grondona, J. M. (1997). Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. *Development* **124,** 313–326.

Kawamura, Y., Fan, Q.-W., Hayashi, H., Michikawa, M., Yanagisama, K., and Komano, H. (1999). Expression of the mRNA for two isoforms of neural plakophorin-related arm-repeat protein/d-catenin in rodent neurons and glial cells. *Neurosci. Lett.* **277,** 185–188.

Kelley, M. W., Xu, X.-M., Wagner, M. A., Warchol, M. E., and Corwin, J. T. (1993). The developing organ of Corti contains retinoic acid and forms supernumerary hair cells in response to exogenous retinoic acid in culture. *Development* **119,** 1041–1053.

Kelley, M. W., Turner, J. K., and Reh, T. A. (1994). Retinoic acid promotes differentiation of photoreceptors *in vitro*. *Development* **120,** 2091–2102.

Kessel, M. (1993). Reversal of axonal pathways from rhombomere 3 correlates with extra *Hox* expression domains. *Neuron* **10,** 379–393.

Kim, T.-A., Lim, J., Ota, S., Raja, S., Rogers, R., Rivnay, B., Avraham, H., and Avraham, S. (1998). NRP/B, a novel nuclear matrix protein, associates with p110RB and is involved in neuronal differentiation. *J. Cell Biol.* **141,** 553–566.

Kliewer, S. A., Umesono, K., Evans, R. M., and Mangelsdorf, D. J. (1994). The retinoid X receptors: Modulators of multiple hormonal signaling pathways. *In* "Vitamin A in health and disease" (R. Blomhoff, Ed.), pp. 239–255. Marcel Dekker Inc., New York.

Knoepfler, P. S., and Kamps, M. P. (1997). The Pbx family of proteins is strongly upregulated by a post-transcriptional mechanism during retinoic acid-induced differentiation of P19 embryonal carcinoma cells. *Mech. Dev.* **63,** 5–14.

Kobayashi, M., Matsuoka, I., and Kurihara, K. (1994a). Cholinergic differentiation of cultured sympathetic neurons induced by retinoic acid. Induction of choline acetyltransferase mRNA and suppression of tyrosine hydroxylase mRNA levels. *FEBS Lett.* **337,** 259–264.

Kobayashi, M., Kurihara, K., and Matsuoka, I. (1994b). Retinoic acid induces BDNF responsiveness of sympathetic neurons by alteration of Trk neurotrophin receptor expression. *FEBS Lett.* **356,** 60–65.

Kobayashi, M., Fujii, M., Kurihara, K., and Matsuoka, I. (1998). Bone morphogenetic protein-2 and retinoic acid induce neurotrophin-3 responsiveness in developing rat sympathetic neurons. *Mol. Brain Res.* **53,** 206–217.

Kojima, N., Kono, M., Yoshida, Y., Tachida, Y., Nakafuku, M., and Tsuji, S. (1996). Biosynthesis and expression of polysialic acid on the neural cell adhesion molecule is predominantly directed by ST8Sia II/STX during *in vitro* neuronal differentiation. *J. Biolog. Chem.* **271,** 22058–22062.

Kolm, P. J., Apekin, V., and Sive, H. (1997). *Xenopus* hindbrain patterning requires retinoid signalling. *Dev. Biol.* **192,** 1–16.

Konig, G., Masters, C. L., and Beyreuther, K. (1990). Retinoic acid induced differentiated neurblastoma cells show increased expression of the bA4 amyloid gene of Alzheimer's disease and an altered splicing pattern. *FEBS Lett.* **269,** 305–310.

Kozireski-Chuback, D., Wu, G., and Leeden, R. W. (1999). Axogenesis in Neuro-2 cells correlates with GMI upregulation in the nuclear and plasma membranes. *J. Neurosci. Res.* **57,** 541–550.

Krezel, W., Ghyselinck, N., Samad, T. A., Dupe, V., Kastner, P., Borelli, E., and Chambon, P. (1998). Imparied locomotion and dopamine signalling in retinoid receptor mutant mice. *Science* **279,** 863–867.

Kuff, E. I., and Fewell, J. W. (1980). Induction of neural-like andacetylcholinesterase activity in cultures of F9 teratocarcinoma cells treated with retinoic acid and dibutyryl cyclic adenosine monophosphate. *Dev. Biol.* **77,** 103–115.

LaMantia, A. S., Colbert, M. C., and Linney, E. (1993). Retinoic acid induction and regional differentiation prefigure olfactory pathway formation in the mammalian forebrain. *Neuron* **10,** 1035–1048.

Lammer, E. J., and Armstrong, D. L. (1992). Malformations of hindbrain structures among humans exposed to isotretinoin (13-*cis*-retinoic acid) during early embryogenesis. *In* "Retinoids in normal development and teratogenesis" (G. Morriss-Kay, Ed.), pp. 281–295. Oxford University Press, Oxford.

Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, A. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Lott, I. T., Richard, J. M., and Sun, S. C. (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313,** 837–841.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M., and Chambon, P. (1995). Mice deficient in cellular retinoic acid binding protein II (CRABP II) or in both CRABP I and CRABPII are essentially normal. *Development* **121,** 539–548.

Langman, J., and Welch, G. W. (1967). Effect of vitamin A on development of the central nervous system. *J. Comp. Neurol.* **128,** 1–16.

Langston, A. W., and Gudas, L. J. (1992). Identification of a retinoic acid responsive enhancer 3′ of the murine homeobox gene Hox-1.6. *Mech. Dev.* **38,** 217–228.

Langston, A. W., and Gudas, L. J. (1994). Retinoic acid and homeobox gene regulation. *Curr. Opin. Genet. Dev.* **4,** 550–555.

Lasorella, A., Iavarone, A., and Israel, M. A. (1995). Differentiation of neuroblastoma enhances Bc1-2 expression and induces alterations of apoptosis and drug resistance. *Cancer Res.* **55,** 4711–4716.

Lee, Y. M., Osumi-Yamashita, N., Ninomiya, Y., Moon, C. K., Eriksson, U., and Eto, K. (1995). Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. *Development* **121,** 825–837.

Lefebvre, P. P., Malgrange, B., Staecker, H., Moonen, G., and van de Water, T. R. (1993). Retinoic acid stimulates regeneration of mammalian auditory hair cells. *Science* **260,** 692–695.

Leonard, L., Horton, C., Maden, M., and Pizzey, J. A. (1995). Anteriorization of CRABP-I expression by retinoic acid in the developing mouse central nervous system and its relationship to teratogenesis. *Dev. Biol.* **168,** 514–528.

Leroy, P., and De Robertis, E. M. (1992). Effects of lithium chloride and retinoic acid on the expression of genes from the *Xenopus Hox 2* complex. *Dev. Dynam.* **194,** 21–32.

Lesma, E., Di Guilio, A. M., Ferro, L., Prino, G., and Gorio, A. (1996). Glycosaminoglycans in nerve injury. I. Low doses of glycosaminoglycans promote neurite formation. *J. Neurosci. Res.* **46,** 565–571.

Levine, J. M., and Flynn, P. (1986). Cell surface changes accompanying the neural differentiation of an embryonal carcinoma cell line. *J. Neurosci.* **6,** 3374–3384.

Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A., and Drager, U. C. (2000). A retinoic acid synthesising enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.* **95,** 283–289.

Li, R., and Ladish, S. (1997). Inhibition of endogenous ganglioside synthesis does not block neurite formation by retinoic acid-treated neuroblastoma cells. *J. Biolog. Chem.* **272,** 1349–1354.

Liesi, P., Rechardt, L., and Wartiovaara, J. (1983). Nerve growth factor induced adrenergic neuronal differentiation in F9 teratocarcinoma cells. *Nature (Lond.)* **306,** 265–267.

Lin, P., Kusano, K., Zhang, Q., Felder, C.-C., Geiger, P. M., and Mahan, L. C. (1996.). GABAa receptors modulate early spontaneous excitatory activity in differentiating P19 neurons. *J. Neurochem.* **66,** 233–242.

Lindberg, C., Wunderlich, M., Ratliff, J., Dinsmore, J., and Jacoby, D. B. (1998). Regulated expression of the homeobox gene, *rPtx2,* in the developing rat. *Dev. Brain Res.* **110,** 215–226.

Linnala, A., Lehto, V. P., and Virtanen, I. (1997). Neuronal differentiation in SH-SY5Y human neuroblastoma cells induces synthesis and kinetics of tenascin and upregulation of av integrin receptors. *J. Neurosci. Res.* **49,** 53–63.

Lipskaia, L., Djiane, A., Defer, N., and Hanoune, J. (1997). Different expression of adenylyl cyclase isoforms after retinoic acid induction of P19 teratocarcinoma cells. *FEBS Lett.* **415,** 275–280.

Liu, H., Nakagawa, T., Kanematsu, T., Uchida, T., and Tsuji, S. (1999). Isolation of 10 differentially expressed cDNAs in differentiated Neuro2a cells induced through controlled expression of the CD3 synthase gene. *J. Neurochem.* **72,** 1781–1790.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A., and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120,** 2723–2748.

Lopez, S. L., and Carrasco, A. E. (1992). Retinoic acid induces changes in the localization of homeobox proteins in the antero-posterior axis of *Xenopus laevis* embryos. *Mech. of Dev.* **36,** 153–164.

Lopez, S. L., Dono, R., Zeller, R., and Carrasco, A. E. (1995). Differential effects of retinoic acid and a retinoid antagonist on the spatial distribution of the homeoprotein Hoxb-7 in vertebrate embryos. *Dev. Dynam.* **204,** 457–471.

Lucarelli, E., Kaplan, D. R., and Thiele, C. J. (1995). Selective regulation of TrkA and TrkB receptors by retinoic acid and interferon-$\gamma$ in human neuroblastoma cell lines. *J. Biolog. Chem.* **270,** 24725–24731.

Luo, J., Pasceri, P., Conlon, R. A., Rossant, J., and Giguere, V. (1995). Mice lacking all isoforms of retinoic acid receptor $\beta$ develop normally and are susceptible to the teratogenic effects of retinoic acid. *Mech. Dev.* **53,** 61–71.

Lyn, S., and Giguere, V. (1994). Localization of CRABP I and CRABP II mRNA in the early mouse embryo by whole-mount *in situ* hybridisation: Implications for teratogenesis and neural development. *Dev. Dynam.* **199,** 280–291.

Lynch, S. A., Brugge, J. S., and Levine, J. M. (1986). Induction of altered c-src product during neuronal differentiation of embryonal carcinoma cells. *Science* **234,** 873–876.

Macdonald, P. N., Bok, D., and Ong, D. E. (1990). Localisation of cellular retinol-binding protein and retinol-binding protein in cells comprising the blood–brain barrier of rat and human. *Proc. Natl. Acad. Sci. USA* **87,** 4265–4269.

MacPherson, P. A., Jones, S., Pawson, P. A., Marshall, K. C., and McBurney, M. W. (1997). P19 cells differentiate into glutaminergic and glutamate-responsive neurons *in vitro*. *Neurosci.* **80,** 487–499.

Maden, M. (1999a). Heads or tails? Retinoic acid will decide. *BioEssays* **21,** 809–812.

Maden, M. (1999b). Retinoids in neural development. *In* "Retinoids: The biochemical and molecular basis of vitamin A and retinoid action" (H. Nau, and W. S. Blaner, Eds.), pp. 399–442. Springer-Verlag, Berlin.

Maden, M., and Holder, N. (1991). The involvement of retinoic acid in the development of the vertebrate central nervous system. *Development* **2**(Suppl.), 87–94.

Maden, M., Ong, D. E., Summerbell, D., Chytil, F., and Hirst, E. A. (1989a). Cellular retinoic acid-binding protein and the role of retinoic acid in the development of the chick embryo. *Dev. Biol.* **135,** 124–132.

Maden, M., Ong, D. E., Summerbell, D., and Chytil, F. (1989b). The role of retinoid-binding proteins in the generation of pattern in the developing limb, the regenerating limb and the nervous system. *Development* (Suppl.), 109–119.

Maden, M., Ong, D. E., and Chytil, F. (1990). Retinoic-binding protein distribution in the developing mammalian nervous system. *Development* **109,** 75–80.

Maden, M., Hunt, P., Eriksson, U., Kuriowa, A., Krumlauf, R., and Summerbell, D. (1991). Retinoic acid-binding protein, rhombomeres and the neural crest. *Development* **111,** 35–44.

Maden, M., Horton, C., Graham, A., Leonard, L., Pizzey, J., Siegenthaler, G., Lumsden, A., and Eriksson, U. (1992a). Domains of cellular retinoic acid-binding protein I (CRABP I) expression in the hindbrain and neural crest of the mouse embryo. *Mech. Dev.* **37,** 13–23.

Maden, M., Gale, E., Horton, C., and Smith, J. C. (1992b). Retinoid-binding proteins in the developing vertebrate nervous system. *In* "Retinoids in normal development and teratogenesis" (G. M. Morriss-Kay, Ed.), pp. 119–134. Oxford University Press, Oxford.

Maden, M., Gale, E., Kostetskii, I., and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Current Biol.* **6,** 417–426.

Maden, M., Graham, A., Gale, E., Rollinson, C., and Zile, M. (1997). Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. *Development* **124,** 2799–2805.

Maden, M., Keen, G., and Jones, G. E. (1998a). Retinoic acid as a chemotactic molecule in neuronal development. *Int. J. Dev. Neurosci.* **16,** 317–322.

Maden, M., Sonneveld, E., van der Saag, P. T., and Gale, E. (1998b). The distribution of endogenous retinoic acid in the chick embryo: Implications for developmental mechanisms. *Development* **125,** 4133–4144.

Maden, M., Gale, E., and Zile, M. (1998c). The role of vitamin A in the development of the central nervous system. *J. Nutr.* **128,** 471S–475S.

Mallo, M., and Brandlin, I. (1997). Segmental identity can change independently in the hindbrain and rhombencephalic neural crest. *Dev. Dynam.* **210,** 146–156.

Manns, M., and Fritzsch, B. (1992). Retinoic acid affects the organization of reticulospinal neurons in developing *Xenopus*. *Neurosci. Lett.* **139,** 253–256.

Mao, Z., and Nadal-Ginard, B. (1996). Functional and physical interactions between mammalian achete-scute homolog 1 and myocyte enhancer factor 2A. *J. Biolog. Chem.* **271,** 14371–14375.

Marayama, K., Usami, M., Aizawa, T., and Yoshikawa, K. (1996). A novel brain-specific mRNA exnoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. *Biochem. Biophys. Res. Commun.* **178,** 291–296.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dolle, P., Gorry, P., Lumsden, A., and Chambon, P. (1993). Two rhombomeres are altered in Hoxa-1 mutant mice. *Development* **119,** 319–338.

Marsh-Armstrong, N., McCaffery, P., Gilbert, W., Dowling, J. E., and Drager, U. C. (1994). Retinoic acid is necessary for development of the ventral retina in zebrafish. *Proc. Natl. Acad. Sci. USA* **91,** 7286–7290.

Marsh-Armstrong, N., McCaffery, P., Hyatt, G., Alonso, L., Dowling, J. E., Gilbert, W., and Drager, U. C. (1995). Retinoic acid in the anteroposterior patterning of the zebrafish trunk. *Roux's Arch. Dev. Biol.* **205,** 103–113.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A., and Krumlauf, R. (1992). Retinoic acid alters hindbrain *Hox* code and induces trasformation of rhombomeres 2/3 into a 4/5 identity. *Nature (Lond.)* **360,** 737–741.

Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuriowa, A., Brenner, S., and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature (Lond.)* **370,** 567–571.

Matsuoka, I., Mizuno, N., and Kurihara, K. (1989). Cholinergic differentiation of clonal rat pheochromocytoma cells (PC12) induced by retinoic acid: Increase of choline acetyltransferase activity and decrease of tyrosine hydroxylase. *Brain Res.* **502,** 53–60.

Matsuoka, I., Kumagai, M., and Kurihara, K. (1997). Differential and coordinated regulation of expression of norepinephrine transporter in catecholaminergic cells in culture. *Brain Res.* **776,** 181–188.

Matsuoka, T., Kondoh, T., Tamaki, N., and Nishizaki, T. (1997). The GABAa receptor is expressed in human neurons derived from a teratocarcinoma cell line. *Biochem. Biophys. Res. Commun.* **237,** 719–723.

McBurney, M. W., Jones-Villeneuve, E. M. V., Edwards, M. K. S., and Anderson, P. J. (1982). Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature (Lond.)* **299,** 165–167.

McBurney, M. W., Reuhl, K. R., Ally, A. I., Nasipuri, S., Bell, J. C., and Craig, J. (1988). Differentiation and maturation of embryonal carcinoma-derived neurons in cell culture. *J. Neurosci.* **8,** 1063–1073.

McCaffery, P., and Drager, U. C. (1994a). High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. *Proc. Natl. Acad. Sci. USA* **91,** 7772–7776.

McCaffery, P., and Drager, U. C. (1994b). Hot spots of retinoic acid synthesis in the developing spinal cord. *Proc. Natl. Acad. Sci. USA* **91,** 7194–7197.

McCaffery, P., and Drager, U. C. (1995). Retinoic acid synthesizing enzymes in the embryonic and adult vertebrate. *In* "Enzymology and molecular biology of carbonyl metabolism 5" (H. Weiner *et al.*, Eds.), pp. 173–183. Plenum Press, New York.

McCaffery, P., Tempst, P., Lara, G., and Drager, U. C. (1991). Aldehyde dehydrogenase is a positional marker in the retina. *Development* **112,** 693–702.

McCaffery, P., Lee, M.-O., Wagner, M. A., Sladek, N. E., and Drager, U. (1992). Asymmetrical retinoic acid synthesis in the dorsoventral axis of the retina. *Development* **115,** 371–382.

McCaffery, P., Posch, K. C., Napoli, J. L., Gudas, L., and Drager, U. C. (1993). Changing patterns of the retinoic acid system in the developing retina. *Dev. Biol.* **158,** 390–399.

McCaffery, P., Wagner, E., O'Neil, J., Petkovich, M., and Drager, U. C. (1999). Dorsal and ventral territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. *Mech. Dev.* **82,** 119–130.

McKay, I. J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A., and Lewis, J. (1994). The deaf *Kreisler* mouse: A hindbrain segmentation mutant. *Development* **120,** 2199–2211.

Meissner, J. D., Naumann, A., Mueller, W. H., and Scheibe, R. J. (1999). Regulation of UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase by retinoic acid in P19 cells. *Biochem. J.* **338,** 561–568.

Mendelsohn, C., Ruberte, E., LeMeur, M., Morriss-Kay, G., and Chambon, P. (1991). Developmental analysis of the retinoic acid-inducible RAR-$\beta$2 promoter in transgenic animals. *Development* **113,** 723–734.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120,** 2749–2771.

Mey, J., and Rombach, N. (1999). Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells *in vitro*. *NeuroReport* **26,** 3573–3577.

Mey, J., McCaffery, P., and Drager, U. C. (1997). Retinoic acid synthesis in the developing chick retina. *J. Neurosci.* **17,** 7441–7449.

Mic, F. A., Molotkov, A., Fan, X., Cuenca, A. E., and Duester, G. (2000). RALDH3, a retinaldehyde dehydrogenase that generates retinoic acid, is expressed in the ventral retina, otic vesicle and olfactory pit during mouse development. *Mech. Dev.* **97,** 227–230.

Mizuno, K., Katagiri, T., Maruyama, E., Hasegawa, K., Ogimoto, M., and Yamura, H. (1997). SHP-1 is induced in neuronal differentiation of P19 embryonic carcinoma cells. *FEBS Lett.* **417,**6–12.

Mizuno, N., Matsuoka, I., and Kurihara, K. (1989). Possible involvements of intracellular $Ca^{2+}$ and $Ca^{2+}$-dependent protein phosphorylation in cholinergic differentiation of clonal rat pheochromocytoma cells (PC12) induced by glioma-conditioned medium and retinoic acid. *Dev. Brain Res.* **50,** 1–10.

Mollard, R., Viville, S., Ward, S. J., Decimo, D., Chambon, P., and Dolle, P. (2000). Tissue-specific expression of retinoic acid receptor isoform transcripts in the mouse embryo. *Mech. Dev.* **94,** 223–232.

Momoi, T., Kitamoto, T., Kasuya-Sato, T., Seno, H., and Momoi, M. (1989). Developmental changes in the expression and distribution of cellular retinoic acid binding protein CRABP in the central nervous system of the chick embryo. *Biomed. Res.* **10,** 43–48.

Momoi, T., Kawai, Y., Momoi, M., and Etoh, Y. (1992). Activin synergistically increased c-jun mRNA in P19 embryonal carcinoma cells in the presence of retinoic acid. *Biochem. Biophys. Res. Commun.* **184,** 1350–1356.

Morishita, R., Shinohara, H., Ueda, H., Kao, K., and Asano, T. (1999). High expression of the G5

isoform of G protein in neuroepithelial cells and its replacement of the g2 isoform during neuronal differentiation in the rat brain. *J. Neurochem.* **73,** 2369–2374.

Moro Balbas, J. A., Gato, A., Alonso Revuelta, M. I., Pastor, J. F., Represa, J. J., and Barbosa, E. (1993). Retinoic acid induces changes in the rhombencephalic neural crest cells migration and extracellular matrix composition in chick embryos. *Teratology* **48,** 197–206.

Morrison, A., Moroni, M. C., Ariza-McNaughton, L., Krumlauf, R., and Mavilio, F. (1996). *In vitro* and transgenic analysis of a human HOXD4 retinoid-responsive enhancer. *Development* **122,** 1895–1907.

Morriss, G. M. (1972). Morphogenesis of the malformations induced in rat embryos by maternal hypervitaminosis A. *J. Anat.* **113,** 241–250.

Morriss, G. M., and Thorogood, P. V. (1978). An approach to cranial neural crest migration and differentiation in mammalian embryos. *In* "Development in mammals" (M. Johnson, Ed.), pp. 363–412. Oxford University Press, Oxford.

Morriss-Kay, G. M., Murphy, P., Hill, R. E., and Davidson, D. R. (1991). Effects of retinoic acid excess on expression of *Hox-2.9* and *Krox-20* and on morphological segementation in the hindbrain of mouse embryos. *EMBO J.* **10,** 2985–2995.

Mueller, W. H., Kleefeld, D., Khattab, B., Meissner, J. D., and Scheibe, R. J. (2000). Effect of retinoic acid on *N*-glycosylation and mRNA stability of the liver/bone/kidney alkaline phosphatase in neuronal cells. *J. Cell Physiol.* **182,** 50–61.

Muller-Husmann, G., Reinhardt, S., Stahle, M., Lang, E., and Maelicke, A. (1994). EX-1, a surface antigen of mouse neuronal progenitor cells and mature neurons. *Dev. Brain Res.* **83,** 262–277.

Muramatsu, H., and Muramatsu, T. (1991). Purification of recombinant midkine and examination of its biological activities: Functional comparison of new heparin binding factors. *Biochem. Biophys. Res. Commun.* **177,** 652–658.

Muramatsu, H., Shirahama, H., Yonezawa, S., Maruta, H., and Muramatsu, T. (1993). Midkine, a retinoic acid-inducible growth/differentiation factor: Immunochemical evidence for the function and distribution. *Dev. Biol.* **159,** 392–402.

Muramatsu, T. (1993). Midkine (MK), the product of a retinoic acid responsive gene, and pleitrophin constitute a new protein family regulating growth and differentiation. *Int. J. Dev. Biol.* **37,** 183–188.

Muto, K., Noji, S., Nohno, T., Koyama, E., Myokai, F., Nishijima, K., Saito, T., and Taniguchi, S. (1991). Involvement of retinoic acid and its receptor $\beta$ in differentiation of motoneurons in chick spinal cord. *Neurosci. Lett.* **129,** 39–42.

Nakamura, Y., Ozaki, T., Ichimiya, S., Nakagawara, A., and Sakiyama, S. (1998). Ectopic expression of DAN enhances the retinoic acid-induced neural differentiation in human neuroblastoma cells. *Biovhem. Biophys. Res. Commun.* **243,** 722–726.

Nakanishi, T., Ishii, K., Fukushima, N., Asanuma, M., Iwata, E., and Ogawa, N. (1996). Expression of mRNA encoding neurotrophic factors and its regulation in a hybrid neuronal cell line. *Biochem. Mol. Biol. Int.* **38,** 763–772.

Nakao, N., Yokote, H., Nakai, K., and Itakura, T. (2000). Promotion of survival and regeneration of nigral dopamine neurons in a rat model of Parkinson's disease implantation of embryonal carcinoma-derived neurons genetically engineered to produce glial cell line-derived neurotrophic factor. *J. Neurosurg.* **92,** 659–670.

Napoli, J. L. (1994). Retinoic acid homeostasis. *In* "Vitamin A in health and disease" (R. Blomhoff, Ed.), pp. 135–188. Marcel Dekker Inc., New York.

Neelands, T. R., Greenfield, L. J., Zhang, J., Turner, R. S., and Macdonald, R. L. (1998). GABAa receptor pharmacology and subtype mRNA expression in human neuronal NT2-N cells. *J. Neurosci.* **18,** 4993–5007.

Neelands, T. R., Zhang, J., and Macdonald, R. L. (1999). GABAa receptors expressed in undifferentiated human teratocarcinoma NT2 cells differ from those expressed by differentiated NT2-N cells. *J. Neursci.* **19,** 7057–7065.

Niederreither, K., McCaffery, P., Drager, U. C., Chambon, P., and Dolle, P. (1997). Restricted expression

and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62,** 67–78.

Niederreither, K., Subbarayan, V., Dolle, P., and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.* **21,** 444–448.

Niederreither, K., Vermot, J., Schubaur, B., Chambon, P., and Dolle, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127,** 75–85.

Ninomiya, Y., Adams, R., Morriss-Kay, G. M., and Eto, K. (1997). Apoptotic cell death in neuronal differentiation of P19 EC cells: Cell death follows reentry into S phase. *J. Cell Physiol.* **172,** 25–35.

Nittenberg, R., Patel, K. J. Y., Krumlauf, R., Wilkinson, D. G., Brickell, P. M., Tickle, C., and Clarke, J. D. W. (1997). Cell movements, neuronal organisation and gene expression in hindbrains lacking morphological boundaries. *Development* **124,** 2297–2306.

Noll, E., and Miller, R. H. (1994). Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord. *Development* **120,** 649–660.

Novak, J. E., Turner, R. S., Agranoff, B. W., and Fisher, S. K. (1999). Differentiated human NT2-N neurons possess a high intracellular content of myo-inositol. *J. Neurochem.* **72,** 1431–1440.

Nurcome, V., Fraser, N., Herlaar, E., and Heath, J. K. (1992). MK: a pluripotential embryonic stem-cell-derived neuroregulatory factor. *Development* **116,** 1175–1183.

Oehrlein, S. A., Maelicke, A., and Herget, T. (1998). Expression of protein kinase C and gene family members is temporally and spatially regulated during neuronal development *in vitro*. *Eur. J. Cell Biol.* **77,** 323–337.

Ogura, T., Nakayama, K., Fujisawa, H., and Esumi, H. (1996). Neuronal nitric oxide synthase expression in neuronal cell differentiation. *Neurosci. Lett.* **204,** 89–92.

Ohkawa, N., Konishi, Y., Shimada, M., Makino, Y., Yoshikawa, S., Mikoshiba, K., and Tamura, T. (1999). Activation of the mouse inositol 1,4,5-triphosphate receptor type 1 promoter by AP-2. *Gene* **229,** 11–19.

Okazawa, H., Shimizu, J., Kamei, M., Imfuku, I., Hamada, H., and Kanazawa, I. (1996). Bc1-2 inhibits retinoic acid-induced apoptosis during the neural differentiation of embryonal stem cells. *J. Cell Biol.* **132,** 955–968.

Osani, T., Watanabe, Y., and Sanai, Y. (1997). Glycolipid sialyltransferases are enhanced during neural differentiation of mouse embryonal carcinoma cells, P19. *Biochem. Biophys. Res. Commun.* **241,** 327–333.

Osumi-Yamashita, N., Noji, S., Nohno, T., Koyama, E., Doi, H., Eto, K., and Taniguchi, S. (1990). Expression of retinoic acid receptor genes in neural crest-derived cells during mouse facial development. *FEBS Lett.* **264,** 71–74.

Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Sapin, V., Chambon, P., and Dolle, P. (1997). *Meis2,* a novel mouse Pbx-related homoebox gene induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Dev. Dynam.* **210,** 173–183.

Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Mattei, M.-G., Dolle, P., and Chambon, P. (1998). Stra3/lefty, a retinoic acid-inducible novel member of the transforming growth factor-B superfamily. *Int. J. Dev. Biol.* **42,** 23–32.

Packer, A. I., Crotty, D. A., Elwell, V. A., and Wolgemuth, D. J. (1998). Expression of the murine *Hoxa4* gene requires both autoregulation and a conserved retinoic acid response element. *Development* **125,** 1991–1998.

Pahlman, S., Ruusala, A. I., Abrahamson, L., Mattsson, M. E. K., and Esscher, T. (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbol ester-induced differentiation. *Cell Differentiation* **14,** 135–144.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121,** 707–720.

Papalopulu, N., and Kintner, C. (1996). A posteriorising factor, retinoic acid, reveals that anteroposterior

patterning controls the timing of neuronal differentiation in *Xenopus* neurectoderm. *Development* **122,** 3409–3418.

Papalopulu, N., Clarke, J. D. W., Bradley, L., Wilkinson, D., Krumlauf, R., and Holder, N. (1991). Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in *Xenopus* embryos. *Development* **113,** 1145–1158.

Parnas, D., and Linial, M. (1995). Cholinergic properties of neurons differentiated from an embryonal carcinoma cell-line (P19). *Int. J. Dev. Neurosci.* **13,** 767–781.

Passalacqua, M., Patrone, M., Picotti, G. B., del Rio, M., Sparatore, B., and Melloni, E., and Pontremoli S. (1998). Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. *Neurosci.* **82,** 1021–1028.

Paterno, G. D., Gillespie, L. L., Julien, J. P., and Skup, D. (1997). Regulation of neurofilament L, M and H gene expression during retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells. *Mol. Brain Res.* **49,** 247–254.

Pauken, C. M., Laborde, J. B., and Bolon, B. (1999). Retinoic acid acts during peri-implantational development to alter axial and brain formation. *Anat. Embryol.* **200,** 645–655.

Paul, G., and Davies, A. M. (1995). Trigeminal sensory neurons require extrinsic signals to switch neurotrophin dependence during the early stages of target field innervation. *Dev. Biol.* **171,** 590–605.

Perez-Castro, A. V., Toth-Rogler, L. E., Wei, L. N., and Nguyen-Huu, M. C. (1989). Spatial and temporal pattern of expression of the cellular retinoic acid-binding protein and the cellular retinol-binding protein during mouse embryogensis. *Proc. Natl. Acad. Sci. USA* **86,** 8813–8817.

Pevny, L. H., Sockanathan, S., Placzek, M., and Lovell-Badge, R. (1998). A role for SOX1 in neural determination. *Development* **125,** 1967–1978.

Pfeffer, P. L., and De Robertis, E. M. (1994). Regional specificity of RARg isoforms in *Xenopus* development. *Mech. Dev.* **45,** 147–153.

Phillip, J., Mitchell, P. J., Malipiero, U., and Fontana, A. (1994). Cell type-specific regulation of expression of transcription factor AP-2 in neuroectodermal cells. *Dev. Biol.* **165,** 602–614.

Pierani, A., Brenner-Morton, S., Chiang, C., and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97,** 903–915.

Pierce, T., Worman, H. J., and Holy, J. (1999). Neuronal differentiation of NT2/D1 teratocarcinoma cells is accompanied by a loss of lamin A/C expression and an increase in lamin B1 expression. *Exp. Neurol.* **157,** 241–250.

Pleasure, S. J., Page, C., and Lee, V. M. Y. (1992). Pure, postmitotic, polarized human neurons derived from NTera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* **12,** 1802–1815.

Plum, L. A., and Clagett-Dame, M. (1996). All-*trans* retinoic acid stimulates and maintains neurite outgrowth in nerve growth factor-supported developing chick embryonic sympathetic neurons. *Dev. Dynam.* **205,** 52–63.

Pombo, P. M., Barettino, D., Ibarrola, N., Vega, S., and Rodriguez-Pena, A. (1999). Stimulation of the myelin basic protein gene expression by 9-*cis*-retinoic acid and thyroid hormone: Activation in the context of its native promoter. *Brain Res. Mol. Brain Res.* **64,** 92–100.

Ponzoni, M., and Lanciotti, M. (1990). Retinoic acid rapidly decreases phosphatidyinositol turnover during neuroblastoma cell differentiation. *J. Neurochem.* **54,** 540–546.

Popperl, H., and Featherstone, M. S. (1993). Identification of a retinoic acid response element upstream of the murine *Hox-4.2* gene. *Mol. Cell Biol.* **13,** 257–265.

Pratt, R. M., Goulding, E. H., and Abbott, B. D. (1987). Retinoic acid inhibits migration of cranial neural crest cells in the cultured mouse embryo. *J. Craniofac. Genet. Dev. Biol.* **7,** 205–217.

Pratt, M. A. C., Kralova, J., and McBurney, M. W. (1990). A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Mol. Cell Res.* **10,** 6335–6453.

Quinn, S. D. P., and De Boni, U. (1991). Enhanced neuronal regeneration by retinoic acid of murine dorsal root ganglia and of fetal murine and human spinal cord *in vitro*. *In Vitro Cell. Dev. Biol.* **27A,** 55–62.

Ramkumar, T., and Adler, S. (1999). A requirement for the POU transcription factor Brn-2, in corticotrophin-releasing hormone expression in a neuronal cell line. *Mol. Endocrinol.* **13,** 1237–1248.

Raulais, D., Lagente-Chevallier, O., Guettet, C., Duprez, D., Courtois, Y., and Vigny, M. (1991). A new heparin binding protein regulate by retinoic acid from chick embryo. *Biochem. Biophys. Res. Commun.* **174,** 708–715.

Raz, Y., and Kelley, M. W. (1999). Retinoic acid signalling is necessary for the development of the organ of Corti. *Dev. Biol.* **213,** 180–193.

Ren, R. F., Lah, J. J., Diehlmann, A., Kim, E. S., Hanver, D. B., Levey, A. I., Beyreuther, K., and Flanders, K. C. (1999). Differential effects of transforming growth factor-bs and glial cell line-derived neurotrophic factor on gene expression of presenilin-1 in human post-mitotic neurons and astrocytes. *Neuroscience* **93,** 1041–1049.

Renoncourt, Y., Carroll, P., Filippi, P., Arce, V., and Alonso, S. (1998). Neurons derived *in vitro* from ES cells express homeoproteins characteristic of motoneurons and interneurons. *Mech. Dev.* **79,** 185–197.

Reynolds, J. N., Prasad, A., Gillespie, L. L., and Paterno, G. D. (1996). Developmental expression of functional GABAa receptors containing the g2 subunit in neurons derived from embryonal carcinoma (P19) cells. *Mol. Brain Res.* **35,** 11–18.

Reynolds, K., Mezey, E., and Zimmer, A. (1991). Activity of the $\beta$-retinoic acid receptor promoter in transgenic mice. *Mech. Dev.* **36,** 15–29.

Riboni, L., Prinetti, A., Bassi, R., Caminiti, A., and Tettamanti, G. (1995). A mediator role of ceramide in the regulation of neuroblastoma Neuro2a cell differentiation. *J. Cell Biol.* **270,** 26868–26875.

Rockwood, J. M., and Maxwell, G. D. (1996a). An analysis of the effects of retinoic acid and other retinoids on the development of adrenergic cells from the avian neural crest. *Exp. Cell Res.* **223,** 250–258.

Rockwood, J. M., and Maxwell, G. D. (1996b). Thyroid hormone decreases the number of adrenergic cells that develop in neural crest cultures and can inhibit the stimulatory action of retinoic acid. *Dev. Brain Res.* **96,** 184–191.

Rodriguez-Tebar, A., and Rohrer, H. (1991). Retinoic acid induces NGF-dependent survival response and high-affinity NGF receptors in immature chick sympathetic neurons. *Development* **112,** 813–820.

Romert, A., Tuvendal, P., Simon, A., Dencker, L., and Eriksson, U. (1998). The identification of a 9-*cis* retinol dehydrogenase in the mouse embryo reveals a pathway for synthesis of 9-*cis* retinoic acid. *Proc. Natl. Acad. Sci. USA* **95,** 4404–4409.

Rosa, F. W., Wilk, A. L., and Kelsey, F. O. (1986). Teratogen update: Vitamin A congeners. *Teratology* **33,** 355–364.

Rossant, J., Zirngibl, R., Cado, D., Shago, M., and Giguere, V. (1991). Expression of a retinoic acid response element-*hsplac*Z transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5,** 1333–1344.

Rossino, P., Defillippi, P., Silengo, L., and Tarone, G. (1991). Up-regulation of the integrin $\alpha 1/\beta 1$ in human nueroblastoma cells differentiated by retinoic acid: Correlation with increased neurite outgrowth responses to laminin. *Cell Reg.* **2,** 1021–1033.

Rowe, A., Eager, N. S. C., and Brickell, P. M. (1991). A member of the RXR nuclear receptor family is expressed in neural-crest-derived cells of the developing chick peripheral nervous system. *Development* **111,** 771–778.

Rowe, A., Richman, J. M., and Brickell, P. M. (1992). Development of the spatial pattern of retinoic acid receptor-$\beta$ transcripts in embryonic chick facial primordia. *Development* **114,** 805–813.

Roy, B., Taneja, R., and Chambon, P. (1995). Synergistic activation of retinoic acid (RA)- responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor $\alpha$ (RAR$\alpha$)-, RAR$\beta$-, or RAR$\gamma$-selective ligand in combination with a retinoid X receptor-specific ligand. *Mol. Cell Biol.* **15,** 6481–6487.

Ruberte, E., Dolle, P., Krust, A., Zelent, A., Morriss-Kay, G., and Chambon, P. (1990). Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development* **108,** 213–222.

Ruberte, E., Dolle, P., Chambon, P., and Morriss-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111,** 45–60.

Ruberte, E., Friederich, V., Morriss-Kay, G., and Chambon, P. (1992). Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. *Development* **115,** 973–987.

Ruberte, E. V. F., Chambon, P., and Morriss-Kay, G. (1993). Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development* **118,** 267–282.

Rubin, W. W., and La Mantia, A.-S. (1999). Age-dependent retinoic acid regulation of gene expression distinguishes the cervical, thoracic, lumbar, and sacral spinal cord regions during development. *Dev. Neurosci.* **21,** 113–125.

Ruiz i Altaba, A., and Jessell, T. (1991a). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. *Genes Dev.* **5,** 175–187.

Ruiz i Altaba, A., and Jessell, T. M. (1991b). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112,** 945–958.

Salvatore, A. M., Cozzolino, M., Gargano, N., Galanti, S., Levi, A., and Alema, S. (1995). Neuronal differentiation of P19 embryonal cells exhibits cell-specific regulation of neurotrophin receptors. *NeuroReport* **6,** 873–877.

Satoh, J., and Kuroda, Y. (2000). Differential gene expression between human neurons and neuronal progenitor cells in culture: An analysis of arrayed cDNA clones in NTera2 human embryonal carcinoma cell line as a model system. *J. Neurosci. Methods* **94,** 155–164.

Satoh, J., Kurohara, K., Yokitake, M., and Kuroda, Y. (1998). Interleukin-15, a T-cell growth factor, is expressed in human neural cell lines and tissues. *J. Neurosci.* **155,** 170–177.

Scheibe, R. J., and Wagner, J. A. (1992). Retinoic acid regulates both the expression of the nerve growth factor receptor and sensitivity to nerve growth factor. *J. Biolog. Chem.* **267,** 17611–17616.

Schuh, T. J., Hall, B. L., Kraft, J. C., Privalsky, M. L., and Kimelman, D. (1993). v-erbA and citral reduce the teratogenic effects of all-*trans*-retinoic acid and retinol, respectively, in *Xenopus* embryogenesis. *Development* **119,** 785–798.

Seegmiller, R. E., Harris, C., Luchtel, D. L., and Juchau, M. R. (1991). Morphological differences elicited by two weak acids, retinoic acid and valproic acid, in rat embryos grown *in vitro*. *Teratology* **43,** 133–150.

Sharma, S., and Notter, M. F. D. (1988). Characterisation of a neurotransmitter phenotype during neuronal differentiation of embryonal carcinoma cells. *Dev. Biol.* **125,** 246–254.

Sharpe, C. R. (1991). Retinoic acid can mimic endogenous signals involved in transformation of the *Xenopus* nervous system. *Neuron* **7,** 239–247.

Sharpe, C. R., and Goldstone, K. (1997). Retinoid receptors promote primary neurogenesis in *Xenopus*. *Development* **124,** 515–523.

Shea, T. B., Fischer, I., and Sapirstein, V. S. (1985). Effect of retinoic acid on growth and morphological differentiation of mouse NB2a neuroblastoma cells in culture. *Dev. Brain Res.* **21,** 307–314.

Shen, S., van den Brink, C. E., Kruijer, W., and van der Saag, P. T. (1992). Embryonic stem cells stably transfected with mRARb2-*lacZ* exhibit specific expression in chimeric embryos. *Int. J. Dev. Biol.* **36,** 465–476.

Shenfelt, R. E. (1972). Morphogenesis of malformations in hamsters caused by retinoic acid: Relation to dose and stage at treatment. *Teratology* **5,** 103–118.

Shiga, T., Guar, V. P., Yamaguchi, K., and Oppenheim, R. W. (1995). The development of interneurons in the chick embryo spinal cord following *in vivo* treatment with retinoic acid. *J. Comp. Neurol.* **360,** 463–474.

Shoji, H., Nakamura, T., van den Eijnden-van Raaji, A. J. M., and Sugino, H. (1998). Identification of a novel type II activin receptor type IIA-N2 induced during the neural differentiation of murine P19 embryonal carcinoma cells. *Biochem. Biophys. Res. Commun.* **246,** 320–324.

Shum, A. S. W., Poon, L. L. M., Tang, W. W. T., Koide, T., Chan, B. W. H., Leung, Y.-C. G., Shiroishi,

T., and Copp, A. J. (1999). Retinoic acid induces down-regulation of Wnt-3a, apoptosis and diversion of tail bud cells to a neural fate in the mouse embryo. *Mech. Dev.* **84,** 17–30.

Sidell, N. (1982). Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells *in vitro*. *J. Natl. Cancer Inst.* **68,** 589–596.

Sidell, N., Altman, A., Haussler, M. R., and Seeger, R. C. (1983). Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Exp. Cell. Res.* **148,** 21–30.

Sidell, N., Lucas, C. A., and Kreutzberg, G. W. (1984). Regulation of acetylcholinesterase acvitity by retinoic acid in a human neuroblastoma cell line. *Exp. Cell Res.* **155,** 305–309.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P. W., Boncinelli, E., and Mavillio, F. (1990). Sequential activation of *HOX2* homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature (Lond.)* **346,** 763–766.

Simeone, A., Acampora, D., Nigro, V., Faiella, A., D'Esposito, M., Stornaiuolo, A., Mavilio, F., and Boncinelli, E. (1991). Differential regulation by retinoic acid of the homeobox genes of the four *Hox* loci in human embryonal carcinoma cells. *Mech. Dev.* **33,** 215–228.

Simeone, A., Avantaggiato, V., Moroni, M. C., Mavilio, F., Arra, C., Cotelli, F., Nigro, V., and Acampora, D. (1995). Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. *Mech. Dev.* **51,** 83–98.

Sive, H. L., and Cheng, P. F. (1991). Retinoic acid perturbs the expression of *Xhox.lab* genes and laters mesodermal determination in *Xenopus laevis*. *Genes Dev.* **5,** 1321–1332.

Sive, H. L., Draper, B. W., Harland, R. M., and Weintrub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. *Genes Dev.* **4,** 932–942.

Smith, S. M. (1994). Retinoic acid receptor isoform $\beta 2$ is an early marker for alimentary tract and central nervous system positional specification in the chicken. *Dev. Dynam.* **200,** 14–25.

Smith, S. M., and Eichele, G. (1991). Temporal and regional differences in the expression pattern of distinct retionic acid receptor-$\beta$ transcripts in the chick embryo. *Development* **111,** 245–252.

Smith-Thomas, L., Lott, I., and Bronner-Fraser, M. (1987). Effects of isotretinoin on the behavior of neural crest cells *in vitro*. *Dev. Biol.* **123,** 276–281.

Smolich, B. D., and Papkoff, J. (1994). Regulated expression of Wnt family members during neuroectodermal differentiation of P19 embryonal carcinoma cells: Overexpression of Wnt-1 perturbs normal differentiation-specific properties. *Dev. Biol.* **166,** 300–310.

Sockanathan, S., and Jessell, T. M. (1998). Motor neuron-derived retinoid signalling specifies the subtype identity of spinal motor neurons. *Cell* **94,** 503–514.

Solomin, L., Johansson, C. B., Zetterstrom, R. H., Bissonnette, R. P., Heyman, R. A., Olson, L., Lehdahl, U., Frisen, J., and Perlmann, T. (1998). Retinoid-X receptor signalling in the developing spinal cord. *Nature (Lond.)* **395,** 398–402.

Sonneveld, E., van den Brink, C. E., van der Leede, B. J., Maden, M., and van der Saag, P. T. (1998). Mouse embryonal carcinoma cell lines stably transfected with retinoic acid receptor B2 promoter-*lacZ*: Sensitive system for measuring levels of active retinoids. *Exp. Cell Res.* **250,** 284–297.

Sonneveld, E., van den Brink, C. E., Tertoolen, L. G. J., van der Burg, B., and van der Saag, P. T. (1999). Retinoic acid hydroxylase (CYP26) is a key enzyme in neuronal differentiation of embryonal carcinoma cells. *Dev. Biol.* **213,** 390–404.

Staines, W. A., Morassutti, D. J., Reuhl, K. R., Ally, A. I., and McBurney, M. W. (1994). Neurons derived from P19 embryonal carcinoma cells have varied morphologies and neurotransmitters. *Neurosci.* **58,** 735–751.

Steller, U., Kohls, S., Muller, B., Soller, R., Muller, R., Schlender, J., and Blohn, D. H. (1996). The RNA biding protein HUD: Rat cDNA and analysis of the alternative spliced mRNA in neuronal differentiating cell lines P19 and PC12. *Mol. Brain Res.* **35,** 285–296.

Stenkamp, D. L., Gregory, J. K., and Adler, R. (1993). Retinoid effects in purified cultures of chick embryo retina neurons and photoreceptors. *Invest. Ophthalmol. Vis. Sci.* **34,** 2425–2436.

Strubing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J., and Wobus, A. M. (1995).

Differentiation of pluripotent embryonic stem cells into the neuronal lineage *in vitro* gives rise to mature inhibitory and excitatory neurons. *Mech. Dev.* **53,** 275–287.

Studer, M., Popperl, H., Marshall, H., Kuriowa, A., and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265,** 1728–1732.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., and Krumlauf, R. (1996). Altered segmental identity and abnormal migration in mice lacking *Hoxb-1*. *Nature (Lond.)* **384,** 630–634.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F. M., Chambon, P., and Krumlauf, R. (1998). Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development* **125,** 1025–1036.

Sulik, K. K., Dehart, D. B., Rogers, J. M., and Shernoff, N. (1995). Teratogenicity of low doses of all-*trans* retinoic acid in presomite mouse embryos. *Teratology* **51,** 398–403.

Sundin, O., and Eichele, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* **114,** 841–852.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M., and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216,** 282–296.

Szujowicz, A., Jankowska, A., Blusztajn, J. K., and Tomaszewicz, M. (1999). Acetylcholine and acetyl-CoA metabolism in differentiating SN56 septal cell line. *J. Neurosci. Res.* **57,** 131–136.

Taira, M., Saint-Jeannet, J.-P., and Dawid, I. B. (1997). Role of the *Xlim-1* and *Xbra* genes in antero-posterior patterning of neural tissue by the head and trunk organiser. *Proc. Natl. Acad. Sci. USA* **94,** 895–900.

Takahashi, J., Palmer, T. D., and Gage, F. H. (1999). Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J. Neurobiol.* **38,** 65–81.

Thang, S. H., Kobayashi, M., and Matsuoka, I. (2000). Regulation of glial cell line-derived neurotrophic factor responsiveness in developing rat sympathetic neurons by retinoic acid and bone morphogenetic protein-2. *J. Neurosci.* **20,** 2917–2925.

Thiele, C. J., Reynolds, C. P., and Israel, M. A. (1985). Decreased expression of N-myc precedes retinoic acid- induced morphological differentiation of human neuroblastoma. *Nature (Lond.)* **313,** 404–406.

Thiele, C. J., Cohen, P. S., and Israel, M. A. (1988). Regulation of c-myb expression in human neuroblastoma cells during retinic acid-induced differentiation. *Mol. Cell Biol.* **8,** 1677–1683.

Thompson, J. N. (1969). Vitamin A in development of the embryo. *Am. J. Clin. Nutr.* **22,** 1063–1069.

Thompson, S., Stern, P. L., Webb, M., Walsh, F. S., Engstrom, W., Evans, E. P., Shi, W.-K., Hopkins, B., and Graham, C. F. (1984). Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic acid. *J. Cell Sci.* **72,** 37–64.

Thorogood, P., Smith, L., Nicol, A., McGinty, R., and Garrod, D. (1982). Effects of vitamin A on the behaviour of migratory neural crest cells *in vitro*. *J. Cell Sci.* **57,** 331–350.

Tokuhiro, S., Tomita, T., Iwata, H., Kosaka, T., Saido, T. C., Murayama, K., and Iwatsubo, T. (1998). The presenilin 1 mutation (M146V) linked to familial Alzheimer's disease attenuates the neuronal differentiation of NTera 2 cells. *Biochem. Biophy. Res. Commun.* **244,** 751–755.

Tokumoto, Y. M., Durand, B., and Raff, M. C. (1999). An analysis of the early events when oligodendrocyte precursor cells are triggered to differentiate by thyroid hormone, retinoic acid or PDGF withdrawal. *Dev. Biol.* **213,** 327–339.

Tonini, R., Mancinelli, E., Balestrini, M., Mazzanti, M., Martegani, E., Ferroni, A., Sturani, E., and Zippel, R. (1999). Expression of Ras-GRF in the SK-N-BE neuroblastoma accelerates retinoic-acid-induced neuronal differentiation and increases the functional expression of the IRK1 potassium channel. *Eur. J. Neurosci.* **11,** 959–966.

Toresson, H., Mata de Urquiza, A., Fagerstrom, C., Perlmann, T., and Campbell, K. (1999). Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. *Development* **126,** 1317–1326.

Trofimova-Griffin, M. E., Brezezinski, M. R., and Juchau, M. R. (2000). Patterns of CYP26 expres-

sion in human prenatal cepahlic and hepatic tissues indicate an important role during early brain development. *Dev. Brain Res.* **120,** 7–16.

Trojanowski, J. Q., Kleppner, S. R., Hartley, R. S., Miyazono, M., Fraser, N. W., Kesari, S., and Lee, V. M. Y. (1997). Transfectable and transplantable postmitotic human neurons: A potential "platform" for gene therapy of nervous system diseases. *Exp. Neurol.* **144,** 92–97.

Tryoen-Toth, P., Gaveriaux-Ruff, C., and Labourdette, G. (2000). Down-regulation of mu-opioid receptor expression in rat oligodendrocytes during their development *in vitro*. *J. Neurosci. Res.* **60,** 10–20.

Turetsky, D. M., Huettner, J. E., Gottlieb, D. I., Goldberg, M. P., and Choi, D. W. (1993). Glutamate receptor-mediated surrents and toxicity in embryonal carcinoma cells. *J. Neurobiol.* **24,** 1157–1169.

Turner, E. E., Jenne, K. J., and Rosenfeld, M. G. (1994). Brn-3.2: A Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid. *Neuron* **12,** 205–218.

Ulven, S., Gundersen, T. E., Weedon, M. S., Landaas, V. O., Sakhi, A. K., Fromm, S. H., Geronimo, B. A., Moskaug, J. O., and Blomhoff, R. (2000). Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of all-*trans*-retinoic acid. *Dev. Biol.* **220,** 379–391.

Vaessen, M.-J., Kootwijk, E., Mummery, C., Hilkens, J., Bootsma, D., and van Kessel, A. D. (1989). Preferential expression of cellular retinoic acid binding protein in a subpopulation of neural cells in the developing mouse embryo. *Differentiation* **40,** 99–105.

Vaessen, M.-J., Meijers, J. H. C., Bootsma, D., and van Kessel, A. D. (1990). The cellular retinoic acid-binding protein is expressed in tissues associated with retinoic acid-induced malformations. *Development* **110,** 371–378.

Vaillant, A. R., and Brewin, D. L. (1995). Accumulation of microtubule-associated protein 1A (MAP1A) in differentiating P19 embryonal carcinoma cells. *Biochem. Cell Biol.* **73,** 695–702.

Valdenaire, O., Maus-Moatti, M., Vincent, J. D., Mallet, J., and Vernier, P. (1998). Retinoic acid regulates the developmental expression of dopamine D2 receptors in rat striatal primary cultures. *J. Neurochem.* **71,** 929–936.

Vallari, R. C., and Pietruszko, R. (1982). Human aldehyde dehydrogenase: mechanism of inhibition by disulphiram. *Science* **216,** 637–639.

van den Eijnden-van Raaij, A. J. M., van Achterberg, T. A. E., van der Kruijssen, C. M. M., Piersma, A. H., Huylebroeck, D., de Laat, S. W., and Mummery, C. L. (1991). Differentiation of aggregated murine P19 embryonal carcinoma cells is induced by a novel visceral endoderm-specific FGF-like factor and inhibited by activin A. *Mech Dev.* **33,** 157–166.

van der Wees, J., Schilthuis, J. G., Koster, C. H., Diesveld-Schipper, H., Folkers, G. E., van der Saag, P. T., Dawson, M. I., Shudo, K., van der Burg, B., and Durston, A. J. (1998). Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. *Development* **125,** 545–556.

van Holst, A., Rogriguez-Tebar, A., Michaille, J.-J., Dhouailly, D., Backstrom, A., Ebendal, T., and Rohrer, H. (1995). Retinoic acid-mediated increase in TrkA expression is sufficient to elicit NGF-dependent survival of sympathetic neurons. *Mol. Cell Neurosci.* **6,** 185–198.

van Holst, A., Lefcort, F., and Rohrer, H. (1997). Trka expression levels of sympathetic neurons correlate with NGF-dependent survival during development and after treatment with retinoic acid. *Eur. J. Neurosci.* **9,** 2166–2177.

van Inzen, W. G., Peppelenbosch, M. P., van den Brand, M. W. M., Terteolen, L. G. J., and de Laat, S. (1996). The role of receptor protein tyrosine phosphatase a in neuronal differentiation of embryonic stem cells. *Dev. Brain Res.* **91,** 304–307.

Ved, H. S., and Pieringer, R. A. (1993). Regulation of neuronal differentiation by retinoic acid alone and in cooperation with thyroid hormone or hydrocortisone. *Dev. Neurosci.* **15,** 49–53.

Vogel, S., Gamble, M. V., and Blaner, W. S. (1999). Biosynthesis, absorption, metabolism and transport

of retinoids. *In* "Retinoids: The biochemical and molecular basis of vitamin A and retinoid action" (H. Nau and W. S. Blaner, Eds.), pp. 31–95. Springer-Verlag, Berlin.

Wagner, E., McCaffery, P., and Drager, U. (2000). Retinoic acid in the formation of the dorsoventral retina and its central projections. *Dev. Biol.* **222,** 460–470.

Wagner, M., Thaller, C., Jessell, T. M., and Eichele, G. (1990). Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature (Lond.)* **345,** 819–822.

Wagner, M., Han, B., and Jessell, T. M. (1992). Regional differences in retinoid release from embryonic neural tissue detected by an *in vitro* reporter assay. *Development* **116,** 55–66.

Wakeman, J. A., Heath, P. R., Pearson, R. C., and Andrews, P. W. (1997). MAL mRNA is induced during the differentiation of human embryonal carcinoma cells into neurons and is also localised within specific regions of the human brain. *Differentiation* **62,** 97–105.

Wakeman, J. A., Walsh, J., and Andrews, P. W. (1998). Human Wnt-13 is developmentally regulated during the differentiation of NTER-2 pluripotent human embryonal carcinoma cells. *Oncogene* **17,** 179–186.

Wang, S.-Y., LaRosa, G. J., and Gudas, L. J. (1985). Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev. Biol.* **107,** 75–86.

Wang, X., and Halvorsen, S. W. (1998). Retinoic acid up-regulates ciliary neurotrophic factor receptors in cultured chick neurons and cardiomyocytes. *Neurosci. Lett.* **240,** 9–12.

Wang, Y.-Z., and Christakos, S. (1995). Retinoic acid regulates the expression of the cacium binding protein, calbindin-D28K. *Mol. Endocrinol.* **9,** 1510–1521.

Warkany, J. (1945). Manifestations of prenatal nutritional deficiency. *Vit. Horm.* **3,** 73–103.

Watanabe, Y., Watanabe, T., Kitagawa, M., Taya, Y., Nakayama, K., and Motoyama, N. (1999). pRb phosphorylation is regulated differentially by cyclin-dependent kinase (Cdk)2 and Cdk4 in retinoic acid-induced neuronal differentiation of P19 cells. *Brain Res.* **842,** 342–350.

Webster, W. S., Johnston, M. C., Lammer, E. J., and Sulik, K. K. (1986). Isotretinoin embryopathy and the cranial neural crest: An *in vivo* and *in vitro* study. *J. Craniofac. Gen. Dev. Biol.* **6,** 211–222.

White, J. A., Guo, Y.-D., Baetz, K., Beckett-Jones, B., Bonasoro, J., Hsu, K. E., Dilworth, F. J., Jones, G., and Petkovich, M. (1996). Identification of the retinoic acid-inducible all- *trans*-retinoic acid 4-hydroxylase. *J. Biolog. Chem.* **271,** 29922–29927.

White, J. A., Beckett-Jones, B., Guo, Y.-D., Dilworth, F. J., Bonasoro, J., Jones, G., and Petkovich, M. (1997). cDNA cloning of human retinoic acid-metabolizing enzyme (hp450RAI) identifies a novel family of cytochromes P450 (CYP26). *J. Biolog. Chem.* **272,** 18538–18541.

White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, H. F., and Clagett-Dame, M. (1998). Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-*trans*-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95,** 13459–13464.

White, J. C., Highland, M., Kaiser, M., and Clagett-Dame, M. (2000). Vitamin A deficiency results in the dose-dependent acquisition of anterior character and shortening of the caudal hindbrain of the rat embryo. *Dev. Biol.* **220,** 263–284.

Wilson, J. G., Roth, C. B., and Warkany, J. (1953). An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am. J. Anat.* **92,** 189–217.

Wion, D., Houlgatte, R., Barbot, N., Barrand, P., Dicou, E., and Brachet, P. (1987). Retinoic acid increases the expression of NGF gene in mouse L cells. *Biochem. Biophys. Res. Commun.* **149,** 510–514.

Wohl, C. A., and Weiss, S. (1998). Retinoic acid enhances neuronal proliferation and astroglial differentiation in cultures of CNS stem cell-derived precursors. *J. Neurobiol.* **37,** 281–290.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. Theor. Biol.* **25,** 1–47.

Wood, H., Pall, G., and Morriss-Kay, G. (1994). Exposure to retinoic acid before or after the onset of

somitogenesis reveals separate effects on rhombomeric segmentation and 3′ *HoxB* gene expression domains. *Development* **120,** 2279–2285.

Wu, G., Fang, Y., Lu, Z. H., and Leeden, R. W. (1998). Induction of axon-like and dendrite-like processes in neuroblastoma cells. *J. Neurocytol.* **27,** 1–14.

Wuarin, L., and Sidell, N. (1991). Differential susceptibilities of spinal cord neurons to retinoic acid-induced survival and differentiation. *Dev. Biol.* **144,** 429–435.

Wuarin, L., Sidell, N., and De Vellis, J. (1990). Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *Int. J. Dev. Neurosci.* **8,** 317–326.

Wuarin, L., Chang, B., Wada, R., and Sidell, N. (1994). Retinoic acid upregulates nuclear retinoic acid receptor-a expression in human neuroblastoma cells. *Int. J. Cancer* **56,** 840–845.

Wulf, P., and Suter, U. (1999). Embryonic expression of epithelial membrane protein 1 in early neurons. *Dev. Brain Res.* **116,** 169–180.

Wyatt, S., Ensor, L., Begbee, J., Ernfors, P., Reichardt, L F., and Latchman, D. S. (1998). NT-3 regulates expression of Brn3a but not Brn3b in developing mouse trigeminal sensory neurons. *Mol. Brain Res.* **55,** 254–264.

Wyatt, S., Andres, R., Rohrer, H., and Davies, A. M. (1999). Regulation of neurotrophin receptor expression by retinoic acid in mouse sympathetic neuroblasts. *J. Neurosci.* **19,** 1062–1071.

Yamamoto, M., McCaffery, P., and Drager, U. C. (1996). Influence of the choroid plexus on cerebellar development: Analysis of retinoic acid synthesis. *Dev. Brain Res.* **93,** 182–190.

Yan, Y.-L., Jowett, T., and Postlethwait, J. H. (1998). Ectopic expression of hoxb2 after retinoic acid treatment or mRNA injections: Disruption of hindbrain and craniofacial morphogenesis in zebrafish embryos. *Dev. Dynam.* **213,** 370–385.

Yang, Y., Quitschke, W. W., and Brewer, G. J. (1998). Upregulation of amyloid precursor protein gene promoter in rat primary hippocampal neurons by phorbol ester, IL-1 and retinoic acid, but not by reactive oxygen species. *Mol. Brain Res.* **60,** 40–49.

Yasuda, Y., Okamoto, M., Konishi, H., Matsuo, T., Kihara, T., and Tanimura, T. (1986). Developmental anomalies induced by all-*trans* retinoic acid in fetal mice: I. Macroscopic findings. *Teratology* **34,** 37–49.

Yasuda, Y., Konishi, H., Matsuo, T., Kihara, T., and Tanimura, T. (1989). Aberrant differentiation of neuroepithelial cells in developing mouse brains subsequent to retinoic acid exposure *in utero*. *Am. J. Anat.* **186,** 271–284.

Yokota, Y., and Ohkubo, H. (1996). 9-*cis*-retinoic acid induces neuronal differentiation of retinoi acid-nonresponsive embryonal carcinoma cells. *Exp. Cell Res.* **228,** 1–7.

Younkin, D. P., Tang, C.-M., Hardy, M., Reddy, U. R., Shi, Q.-Y., Pleasure, S. J., Lee, V. M. Y., and Pleasure, D. (1993). Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. *Proc. Natl. Acad. Sci. USA* **90,** 2174–2178.

Zeidman, R., Lofgren, B., Pahlman, S., and Larsson, C. (1999). PKCe, via its regulatory domain and independently of its catalytic domain, induced neurite-like processes in neuroblastoma cells. *J. Cell Biol.* **145,** 713–726.

Zgombic-Knight, M., Ang, H. L., Foglio, M. H., and Duester, G. (1995). Cloning of the mouse class IV alcohol dehydrogenase (retinol dehydrogenase) cDNA and tissue-specific expression patterns of the murine ADH gene family. *J. Biolog. Chem.* **270,** 10868–19877.

Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R., and Grippo, J. F. (1994). Ectopic *Hoxa-1* induces rhombomere transformation in mouse hindbrain. *Development* **120,** 2431–2442.

Zhang, Z., Balmer, J. E., Lovlie, A., Fromm, S. H., and Blomhoff, R. (1996). Specific teratogenic effects of different retinoic acid isomers and analogs in the developing anterior central nervous system of zebrafish. *Dev. Dynam.* **206,** 73–86.

Zhao, D., McCaffery, P., Ivins, K. J., Neve, R. L., Hogan, P., Chin, W. W., and Drager, U. C. (1996). Molecular identification of a major retinoic acid-synthesising enzyme, a retinaldehyde-specific dehydrogenase. *Eur. J. Biochem.* **240,** 15–22.

This Page Intentionally Left Blank

# The Roles of Receptor-Associated Protein (RAP) as a Molecular Chaperone for Members of the LDL Receptor Family

Guojun Bu
Departments of Pediatrics, and of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Members of the LDL receptor family mediate endocytosis and signal transduction of many extracellular ligands which participate in lipoprotein metabolism, protease regulation, embryonic development, and the pathogenesis of disease (e.g., Alzheimer's disease). Structurally, these receptors share common motifs and modules that are highlighted with clusters of cysteine-rich ligand-binding repeats. Perhaps, the most significant feature that is shared by members of the LDL receptor family is the ability of a 39-kDa receptor-associated protein (RAP) to universally inhibit ligand interaction with these receptors. Under physiological conditions, RAP serves as a molecular chaperone/escort protein for these receptors to prevent premature interaction of ligands with the receptors and thereby ensures their safe passage through the secretory pathway. In addition, RAP promotes the proper folding of these receptors, a function that is likely independent from its ability to inhibit ligand binding. The molecular mechanisms underlying these functions of RAP, as well as the molecular determinants that contribute to RAP–receptor interaction will be discussed in this review. Elucidation of these mechanisms should help to clarify how a specialized chaperone promotes the biogenesis of LDL receptor family members, and may provide insights into how the expression and function of these receptors can be regulated via the expression of RAP under pathological states.

**KEY WORDS:** RAP, Endocytosis receptors, LDL receptor family, LRP, Megalin, Chaperone, Protein folding, Protein trafficking. 

## I. Introduction

The low-density lipoprotein receptor (LDLR) family is one of the fastest growing receptor families we have witnessed during the past decade. Our understanding of the functions of these receptors has expanded from receptor-mediated endocytosis to signal transduction at the cellular level and from lipoprotein metabolism to blood coagulation, embryonic development, and various disease-related mechanisms at the physiological and pathophysiological levels (Hussain *et al.,* 1999; Krieger and Herz, 1994; Willnow, 1999). Structurally, all members of the LDLR family are single-transmembrane receptors and contain, in their ectodomains, clusters of cysteine-rich ligand-binding repeats (also called complement-type repeats) and epidermal growth factor (EGF) precursor homology domains consisting of multiple copies of EGF-like repeats as well as regions with YWTD consensus sequences. The complex structure of the LDLR family members is highlighted by the structure of the ligand-binding repeats, each of which consists of approximately 40 amino acids that include six cysteine residues, forming three disulfide bridges. These disulfide bonds, together with an octahedral calcium cage within each repeat, are believed to be essential for stabilization of the structure of individual ligand-binding repeats.

The complex structures of the LDLR family members suggest that the biosynthesis of these receptors may require the assistance of molecular chaperones. Indeed, studies with the LDLR-related protein (LRP), megalin, and more recently the very-low-density lipoprotein receptor (VLDLR), have shown that a receptor-associated protein (RAP) serves as a specialized chaperone for these receptors. RAP was initially found to copurify with LRP, and was later found to antagonize ligand binding to the receptor. However, the physiological function of RAP was not appreciated until its subcellular localization was revealed to be within the early secretory pathway (i.e., the endoplasmic reticulum (ER) and Golgi). Both cellular and genetic studies have shown that RAP is required for proper folding and subsequent trafficking of LRP, megalin, the VLDLR, and likely other members of the LDLR family. When compared to other general ER chaperones (e.g., BiP and calnexin), the unique features of RAP include its specificity to members of the LDLR family, and its dual roles in receptor folding and trafficking. In this review, I will discuss the function of RAP as a molecular chaperone for members of the LDLR family, and propose mechanisms as to how RAP interacts with these receptors and displays its function. Since the function of RAP as a molecular chaperone was defined primarily with LRP and megalin, I will focus on studies utilizing these two receptors as the target receptors.

## II. LDLR Family

The LDLR family was recognized as several new endocytic receptors that share structural homology with the LDLR were discovered during the past decade. The current number of members of this family in mammals has grown to over 10 receptors and, in addition to the LDLR itself, include LRP (Herz *et al.,* 1988), megalin (Raychowdhury *et al.,* 1989; Saito *et al.,* 1994), the VLDLR (Takakashi *et al.,* 1992), apoE receptor 2 (apoER2)/LR8B (Brandes *et al.,* 1997; Kim *et al.,* 1996; Novak *et al.,* 1996), sorLA-1/LR11 (Jacobsen *et al.,* 1996; Yamazaki *et al.,* 1996), LRP3 (Ishii *et al.,* 1998), LRP4 (Tomita *et al.,* 1998), LRP5 (Hey *et al.,* 1998; Kim *et al.,* 1998), LRP6 (Brown *et al.,* 1998), and LRP1B/LRP-DIT (Liu *et al.,* 2000). Among these receptors, LRP, megalin, and LRP1B are large receptors ($M_r \geq 600$ kDa), each containing four clusters of ligand binding repeats, whereas the other members are smaller in size and contain only one or two clusters of ligand-binding repeats. A model depicting the major structural components of these receptors is shown in Fig. 1.

All members of the LDLR family, except for LRP4, are type I transmembrane glycoproteins with cytoplasmic tails containing tyrosine-based endocytosis signals. The most distinctive structural components of the LDLR family are the cysteine-rich ligand-binding repeats, which form the ligand-binding domains. These ligand-binding repeats, each of which is ~40 amino acids in length and contains six cysteine residues, are also found in a number of complement components and are therefore also referred to as complement-type repeats (CR). The three-dimensional structures of three LDLR repeats (Daly *et al.,* 1995a,b; Fass *et al.,* 1997), and one LRP repeat (Huang *et al.,* 1999), have been solved via either protein crystallization or NMR analysis. These structural analyses show that, in addition to the three disulfide bridges, each of the ligand-binding repeat contains a $Ca^{2+}$ ion, which forms an octagonal structure with neighboring acidic amino acid residues. It appears that several mutations within the LDLR, which had previously been thought to disrupt ionic interaction with its ligands, are likely to cause structural defects within the receptor due to disruption of the $Ca^{2+}$ cage (Fass *et al.,* 1997). In addition to the ligand-binding repeats, most members of the LDLR gene family also contain EGF-like repeats and YWTD motifs. Furthermore, a region that contains a cluster of O-linked glycosylation is present in some of these receptors.

In addition to common structural modules, there are also functional similarities among various members of the LDLR family. For example, several members of the family (LRP, megalin, and the VLDLR) bind multiple ligands, and thus are defined as multifunctional receptors. One of the ligands, apolipoprotein E

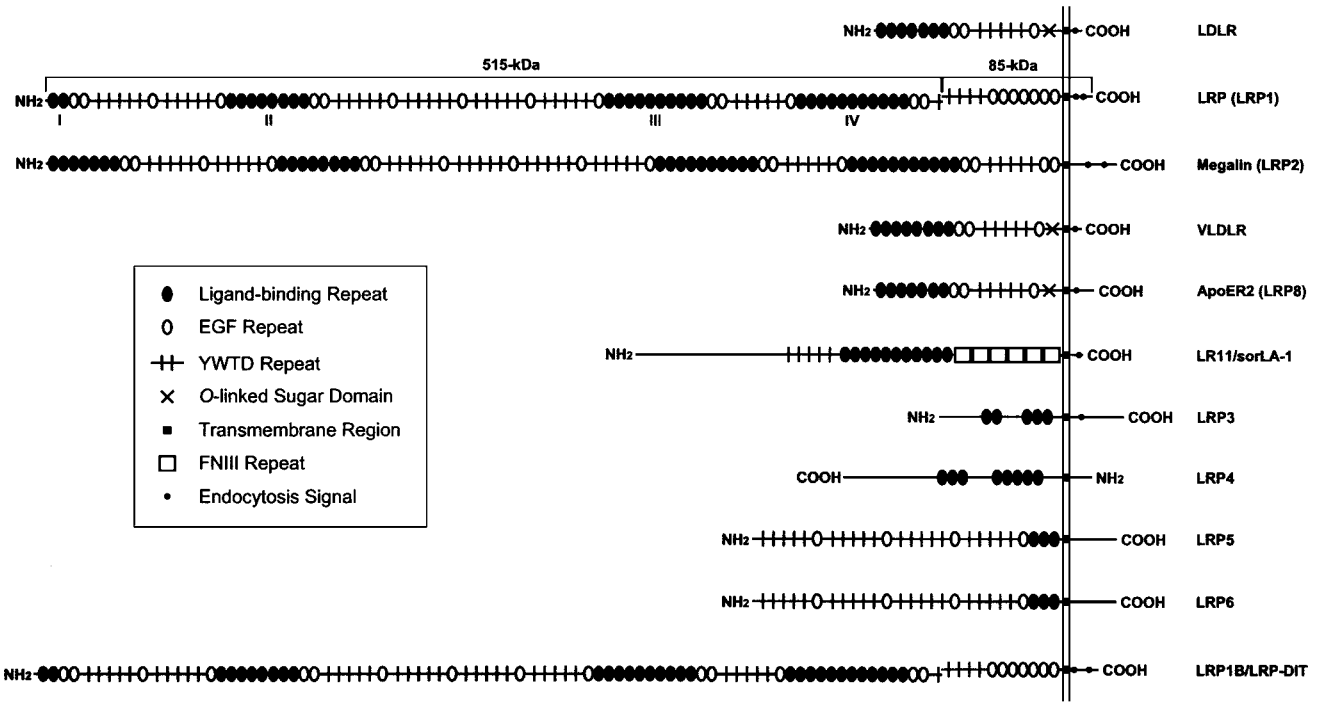



FIG. 1 The LDLR family. Schematic representations of LDLR family members in mammals. Important structural features are illustrated (see text). The two furin-cleaved subunits and the four clusters of ligand-binding repeats of LRP are marked. These receptors and their structural modules are drawn approximately to actual scales.

(apoE)-containing lipoprotein, binds most of the receptors in the family. Thus, many of these receptors may be involved in lipid metabolism. However, there are also ligands that bind only to one of these receptors. For example, protease- or methylamine-activated $\alpha_2$-macroglobulin ($\alpha_2M^*$) binds only to LRP, but not to other members of the family. Furthermore, the functions of each of these receptors can be distinguished by their specific tissue expression (see below).

Recently, several independent studies have demonstrated roles for LDLR family members in cellular signaling wherein they act as transducers of extracellular signals (Herz *et al.,* 2000). For example, cellular signaling through the VLDLR and/or apoER2 is important for the reelin/disabled pathway that participates in neuronal cell migration during embryonic development (Trommsdorff *et al.,* 1999). In addition, LRP6 has recently been shown to be required for the Wnt signaling pathway during embryonic development (Pinson *et al.,* 2000; Tamai *et al.,* 2000; Wehrli *et al.,* 2000). Furthermore, a set of cytoplasmic adaptor and scaffold proteins containing PID or PDZ domains, including mammalian Disabled-1 (mDab1), mDab2, FE65, JNK-interacting protein JIP-1 and JIP-2, PSD-95, CAPON, and SEMCAP-1, bind to the cytoplasmic tails of members of the LDLR family (Gotthardt *et al.,* 2000; Howell *et al.,* 1999; Oleinikov *et al.,* 2000; Stockinger *et al.,* 2000; Trommsdorff *et al.,* 1998, 1999). Thus, taken together these observations expand the functions of these receptors from endocytosis to signal transduction, and from ligand metabolism to embryonic development. Undoubtedly, the regulation of these receptors via molecular chaperones during biogenesis is crucial for their function under physiological and pathophysiological conditions.

## A. LDLR

Lipoprotein metabolism is a major pathway by which humans absorb, store, transport, and utilize lipid components from the diet and maintain lipid homeostasis both in the peripheral circulation system and within the central nervous system (CNS). The transport of lipophilic lipid molecules in an aqueous environment is mediated by lipoprotein particles, which are typically spherical complexes of lipids and apolipoproteins, the latter of which are recognized for cellular uptake by cell surface lipoprotein receptors. The prototypic receptor that mediates the cellular uptake of cholesterol-bearing LDL particles was discovered and characterized by the pioneering work of Brown and Goldstein (Brown and Goldstein, 1986; Goldstein *et al.,* 1985). This receptor mediates cellular uptake of LDL via receptor-mediated endocytosis, a cellular process unveiled largely by the study of the LDLR. Structurally, the LDLR is composed of five structural and functional domains: a ligand-binding domain that contains seven complement-type ligand-binding repeats, an EGF precursor homologous domain, an O-linked glycosylation domain, a membrane-spanning domain, and a relatively short (50 amino

acids) cytoplasmic tail (Brown and Goldstein, 1986; Goldstein *et al.,* 1985). The cytoplasmic tail of the LDLR contains a NPxY sequence, which serves as an endocytosis signal (Chen *et al.,* 1990). Various mutations of the LDLR cause a common metabolic disease termed familial hypercholesterolemia (FH; Brown and Goldstein, 1986). In this disorder, there is an increase in the circulating level of apoB-containing LDL, due to either an absence of or nonfunctional LDLR. Studies of many of the different mutations in LDLR found in FH patients have generated great insights into the structural and functional relationship of the LDLR (Brown and Goldstein, 1986; Hobbs *et al.,* 1990).

## B. LRP

LRP (also known as LRP1) is one of the largest cell surface receptors identified to date (~600 kDa; Herz *et al.,* 1988; Krieger and Herz, 1994). Its extracellular domain structurally resembles that of four combined LDLR molecules and contains 31 ligand-binding repeats grouped into four clusters of 2, 8, 10, and 11 repeats. These four clusters of ligand-binding repeats are separated by EGF precursor homology domains, which contain EGF-like repeats and YWTD motifs. Similar to the LDLR, the extracellular domain precedes a single membrane-spanning segment, which is followed by a cytoplasmic tail. The cytoplasmic tail of LRP (100 amino acids) is twice as long as the tail of the LDLR. Although two copies of the NPxY motif are present within the LRP tail, recent studies from our laboratory have shown that the primary endocytosis signal for LRP are a YxxL motif and a di-leucine motif, but not the two NPxY signals (Li *et al.,* 2000). Another large receptor that shares high structural homology with LRP (see Fig. 1), named LRP1B or LRP-DIT, has recently been identified and found to be frequently deleted in non-small-cell lung cancer cell lines (Liu *et al.,* 2000). However, the physiological ligands and function of this new receptor remain to be defined.

LRP was initially identified by screening a murine lymphocyte cDNA library with an oligonucleotide derived from the ligand-binding motif of the LDLR (Herz *et al.,* 1988). It was subsequently purified from rat liver by immuno-affinity chromatography. Biochemical studies have shown that LRP is synthesized as a single polypeptide chain of approximately 600 kDa and is cleaved by furin in the *trans*-Golgi into two subunits of 515 and 85 kDa, which remain associated with one another as they mature to the cell surface (Herz *et al.,* 1990; Willnow *et al.,* 1996). The 515-kDa amino-terminal subunit binds ligands and remains attached to the membrane through noncovalent association with the 85-kDa transmembrane subunit (Herz *et al.,* 1990; see Fig. 1).

One of the intriguing characteristics of LRP is its ability to bind and endocytose a large array of functionally distinct ligands (Hussain *et al.,* 1999; Krieger and Herz, 1994; Strickland *et al.,* 1995; Willnow, 1999). These ligands share little

sequence homology, yet each can specifically interact with LRP with high affinity. The first ligand identified for LRP was apoE-enriched $\beta$-VLDL (Beisiegel *et al.,* 1989; Kowal *et al.,* 1989), thus suggesting a role for LRP in lipid metabolism. The first evidence of the multiligand nature of LRP came from studies on a receptor for $\alpha_2$M* (Ashcom *et al.,* 1990). It was shown that the peptide sequences of proteolytic fragments of this $\alpha_2$M* receptor were identical to sequences within LRP (Kristensen *et al.,* 1990; Strickland *et al.,* 1990), indicating that LRP is also the receptor for $\alpha_2$M*. The role for LRP in the metabolism of protease, protease/inhibitor complexes was recognized when LRP was shown to be the receptor for tissue-type plasminogen activator (tPA) (Bu *et al.,* 1992a,b), as well as for the complexes of tPA and plasminogen activator inhibitor type I (PAI-1) (Bu *et al.,* 1993; Orth *et al.,* 1992). Other LRP ligands identified to date include complexes of urokinase-plasminogen activator (uPA) and PAI-1, lipoprotein lipase complexed with $\beta$-VLDL, *Pseudomonas* exotoxin A, lactoferrin, tissue factor pathway inhibitor (TFPI), thrombospondin, two forms of $\beta$-amyloid precursor protein (APP) that contain the Kuntiz protease inhibitor (KPI) domain, sphingolipid activator proteins (SAP), several serpin:enzyme complexes (Hussain *et al.,* 1999; Krieger and Herz, 1994; Strickland *et al.,* 1995; Willnow, 1999), and most recently discovered midkine (Muramatsu *et al.,* 2000) and matrix metallproteinase 2 (MMP2, Yang *et al.,* 2001). This diversity of LRP ligands suggests that this receptor may be involved in many different biological processes. Thus, the physiological functions of LRP may be extrapolated from those of its ligands, which include lipid metabolism, regulation of extracellular proteases, blood coagulation/fibrinolysis, cell migration, neuronal development and regeneration, and the pathogenesis of Alzheimer's disease (AD). The broad function of LRP suggest that this receptor is indispensable. Indeed, disruption of the LRP gene resulted in a variety of abnormalities during early embryonic development and ultimate lethality (Herz *et al.,* 1992, 1993). The importance of LRP during early embryonic development may relate to the catabolism of one or more of its ligands and/or its function in bridging important signaling pathways that are not currently defined. Toward the latter possibility, it is interesting to note that several cytosolic adaptor proteins, including FE65, mDab1, JIP-1, JIP-2, and PSD-95, have been shown to bind the cytoplasmic tail of LRP (Gotthardt *et al.,* 2000; Trommsdorff *et al.,* 1998, 1999). In addition, ligand interaction with LRP can result in an increase of intracellular cAMP levels and in the activity of cAMP-dependent protein kinase (PKA,Goretzki and Mueller, 1998; Zhuo *et al.,* 2000). Futhermore, ligation of LRP with antibodies elevates intracellular calcium and inositol 1,4,5-trisphosphate in macrophages (Misra *et al.,* 1999). Taken together, these studies suggest that LRP is also a signaling receptor, although the exact physiological significance of LRP-mediated signaling is not clear at present. Finally, recent studies in our laboratory have shown that the cytoplasmic tail of LRP is phosphorylated at a serine residue by PKA, and that this phosphorylation regulates LRP endocytosis (Li *et al.,* 2001).

## C. Megalin

Megalin (also known as LRP2 or gp330) is another large member of the LDLR family (Christensen *et al.,* 1998; Hussain *et al.,* 1999). It was initially identified as a target antigen for circulating antibodies in Heymann nephritis (Raychowdhury *et al.,* 1989). Similar to LRP's extracellular structure, megalin contains 36 copies of ligand-binding repeats group into four clusters of 7, 8, 10, and 11 (see Fig. 1). The cytoplasmic tail of megalin is twice as long as that of LRP and contains two putative internalization signals (NPxY-based) and several Src-homology binding regions and potential phosphorylation sites, suggesting a potential role for this receptor in signal transduction. Megalin also binds a large number of structurally and functionally distinct ligands, many of which overlap with those of LRP (see Christensen *et al.,* 1998; Hussain *et al.,* 1999 for reviews). However, at least two ligands, apolipoprotein J (apoJ)/clusterin and apolipoprotein B (apoB), bind only to megalin but not to LRP (Kounnas *et al.,* 1995; Stefansson *et al.,* 1995), suggesting the existence of a unique role for megalin in lipoprotein metabolism. Additionally, the tissue expression pattern of megalin is distinct from that of LRP; i.e., megalin is expressed most abundantly in the kidney, whereas LRP is highly expressed in the liver and brain. At the cellular level, megalin is expressed primarily on the apical side of the absorptive epithelial cells. This cellular expression pattern suggests that it may play an important role in the absorption of its ligand by the intestine, the kidney, and the blood–brain barrier. Indeed, megalin has been shown to be involved in the reabsorption of vitamins in the kidney (Moestrup *et al.,* 1998; Nykjaer *et al.,* 1999), the release and renal uptake of thyroid hormones (Marino *et al.,* 2000; Sousa *et al.,* 2000), and the binding and trafficking of cubilin, a 460-kDa protein that associates with intrinsic factor/vitamin $B_{12}$ complexes and albumin (Birn *et al.,* 2000; Hammad *et al.,* 2000; Zhai *et al.,* 2000). Megalin also plays an important role during embryonic development as disruption of megalin gene results in abnormalities in epithelial tissues in mice, which die perinatally from respiratory insufficiency (Willnow *et al.,* 1996). In brain, defective forebrain development that mimics the holoprosencephalic syndrome was observed in megalin-knockout mice, suggesting an important role for megalin in maternal–fetal lipoprotein transport (Willnow *et al.,* 1996).

## D. VLDLR, ApoER2, and Other Members

The VLDLR shares structural similarities with the LDLR, except for the presence of eight ligand-binding repeats as compared to seven present within the LDLR (see Fig. 1; Takakashi *et al.,* 1992). Although originally named the VLDLR, the role of this receptor in lipoprotein metabolism *in vivo* remains to be defined. Studies by Frykan *et al.* (1995) have demonstrated normal levels of plasma lipoproteins in mice homozygous for a disruption in the VLDLR gene, raising the possibility

that the normal function of the VLDLR may be to internalize molecules other than lipoproteins. Indeed over the past several years, the VLDLR has been shown to bind and/or endocytose several ligands that play roles other than lipoprotein metabolism. Many of these ligands overlap with those of LRP and megalin (e.g., protease/protease inhibitor complexes; see Nimpf and Schneider, 1998). However, recently identified VLDLR ligands such as reelin (Hiesberger *et al.,* 1999; Trommsdorff *et al.,* 1999), which also binds to the apoER2 (see below), suggest a unique role of this receptor in neuronal development. Thus, the VLDLR is a multifunctional receptor, a feature that distinguishes this receptor from the LDLR, which binds only apoB- or apoE-containing lipoproteins (Goldstein *et al.,* 1985; Nimpf and Schneider, 1998). Unlike LRP, the VLDLR is highly expressed in heart, muscle, adipose tissue, and brain, but virtually absent from the liver (Takakashi *et al.,* 1992; Webb *et al.,* 1994). Although, the precise significance of VLDLR expression remains to be defined, this distinct tissue distribution for the VLDLR suggests a unique role for this receptor in the metabolism of extracellular ligands in the cardiovascular system and in the CNS.

ApoER2 shows high homology to both the LDLR and the VLDLR, including the positions of the exon/intron boundaries of the genes (Kim *et al.,* 1996). Similar to the LDLR, the apoER2 contains seven copies of ligand-binding repeats. The most notable difference between the tail of apoER2 and that of LDLR or VLDLR is the presence of an additional 59-amino-acid residue encoded by an additional exon (Kim *et al.,* 1996). This domain has been recently reported to interact with two members of the JNK-interacting protein family, JIP-1 and JIP-2, which belong to a group of MAP kinase scaffolding proteins (Gotthardt *et al.,* 2000; Stockinger *et al.,* 2000). The interaction with JIPs is specific for apoER2, since neither the LDLR nor the VLDLR bind these cytosolic proteins (Stockinger *et al.,* 2000). These results suggest that the apoER2 may possess a unique role in intracellular signal transduction.

In addition to LRP, the apoER2 and the VLDLR are also highly expressed in the brain (Kim *et al.,* 1996; Takakashi *et al.,* 1992). However, the significance of this expression was not clear until a recent study byTrommsdorff *et al.* (1999), which showed that double knockout of the VLDLR and the apoER2 in mice results in a defect in neuronal migration during mouse development. This phenotype mimics those of reelin or mDab 1 deficiency. Subsequently, it was demonstrated that reelin is a ligand for both the VLDLR and the apoER2 (D'Arcangelo *et al.,* 1999; Hiesberger *et al.,* 1999), and induces tyrosine phosphorylation of mDab 1 upon binding to these lipoprotein receptors. Since the knockout of the VLDLR or the apoER2 alone produced little if any phenotypic changes, these studies suggest that these two receptors may play backup roles for one another in bridging the signaling pathway from reelin to mDab 1 phosphorylation and neuronal migration.

Several new members of the LDLR family with distinct structural arrangements have been described within the past 2 years (see Fig. 1). These receptors often contain clusters that include only two to five copies of the ligand-binding repeats

(Brown *et al.,* 1998; Ishii *et al.,* 1998; Kim *et al.,* 1998; Tomita *et al.,* 1998). Interestingly, LRP4 is likely a type II transmembrane protein with its amino terminus localized within the cytoplasm (Tomita *et al.,* 1998). The physiological functions of these receptors are largely unclear at present. However, recent studies have shown that LRP6 may function as a coreceptor for Wnt signal transduction (Pinson *et al.,* 2000; Tamai *et al.,* 2000; Wehrli *et al.,* 2000). Most noticeably, mouse embryos homozygous for an insertion mutation in the LRP6 gene exhibit developmental defects that are a striking composite of those caused by mutations in individual Wnt genes (Pinson *et al.,* 2000). The extracellular domain of LRP6 appears to bind Wnt-1 and associate with Fz in a Wnt-dependent manner (Tamai *et al.,* 2000). These new studies further extend the role of LDLR members in embryonic development and suggest that the physiological ligands for some members of the LDLR family are yet to be identified.

## III. RAP Is a Universal Antagonist for Members of the LDLR Family

### A. Discovery of RAP

Although LRP was cloned and described in 1988 by Herz *et al.* (1988), it was not recognized as a receptor for $\alpha_2$M* until 2 years later (Kristensen *et al.,* 1990; Strickland *et al.,* 1990). The original studies on the $\alpha_2$M* receptor were focused on biochemical purification of the receptor from human placenta via affinity chromatography (Ashcom *et al.,* 1990; Jensen *et al.,* 1989). In addition to the two LRP subunits, it was found that a third protein band with molecular size of ~39–40 kDa was copurified with the receptor. The nature of this protein was uncertain until the cDNA for this protein was cloned using an antibody made against the purified receptor complex (Strickland *et al.,* 1991). The new protein was found to be distinct from that of LRP (Herz *et al.,* 1988). In some early studies, this protein was refferred to as the "39-kDa protein" due to its molecular size and largely unknown function. It is now uniformly termed RAP. Human RAP, which contains 323 amino acids (Strickland *et al.,* 1991), was found to be the homolog of a previously identified mouse protein termed heparin-binding protein 44 (HBP-44) (Furukawa *et al.,* 1990), and also the homolog of a rat protein that was identified in kidney as a component of the glycoprotein 330/44-kDa Heymann nephritis antigenic complex (which we now know is the megalin/RAP complex; see Pietromonaco *et al.,* 1990). The major structural features of RAP include a signal peptide that directs the translated RAP into the lumen of the ER, a leucine zipper motif near the amino terminus of the protein with unknown function, a putative N-linked glycosylation site, and a carboxyl-terminal tetrapeptide (HNEL) that is similar to the ER-retention consensus sequence (KDEL). The 120-residue

carboxyl-terminal region of RAP exhibits 26% identity with a region of apoE that includes the LDLR binding domain. This homology with apoE may have both structural and functional significance in regards to binding to receptors and heparin (see below). RAP was also found to bind calmodulin and is phosphorylated by calmodulin-dependent kinase II (Petersen *et al.,* 1996). However, the function of RAP phosphorylation is currently unknown. RAP is ubiquitously expressed in all human tissues and cells examined to date with the highest expression seen in the kidney and brain (Bu *et al.,* 1994b; Zheng *et al.,* 1994). During development, RAP is expressed very early as it has been found in mouse blastocyst (Herz *et al.,* 1992) and in several embryonic cell lines (Czekay *et al.,* 1995; Furukawa *et al.,* 1990).

## B. RAP Universally Antagonizes Ligand Binding to Members of the LDLR Family

The fact that RAP copurifies with LRP suggests that RAP may in some way interact with and regulate LRP. Indeed, Herz *et al.* (1991) showed that RAP, when produced in bacteria as a glutathione *S*-transferase (GST)-fusion protein, is capable of binding to the 515-kDa subunit of LRP on ligand blots. The most dramatic feature of RAP's binding to LRP is its ability to inhibit the subsequent binding and/or uptake of all ligands to LRP. This feature of RAP was first described in two separate reports. The first study (Moestrup and Gliemann, 1991), using RAP purified from human placenta via heparin–Sepharose and a Mono-S cation exchange column, showed that RAP blocks the binding of $\alpha_2$M* to LRP immobilized on nitrocellulose filters in a dose-dependent manner. In the studies by Herz *et al.* (1991), it was found that binding of recombinant GST-RAP to LRP on fibroblasts inhibits the uptake and degradation of apoE-enriched $\beta$-VLDL and $\alpha_2$M*, two of the known ligands for LRP. The ability of RAP to inhibit additional LRP ligands was subsequently demonstrated in numerous studies (Bu *et al.,* 1992b; Kounnas *et al.,* 1992; Nykjaer *et al.,* 1992; Orth *et al.,* 1992; Williams *et al.,* 1992; Willnow *et al.,* 1992). This ability of RAP to universally inhibit LRP ligands distinguishes this protein from other LRP ligands, which do not categorically inhibit the binding and/or uptake of other ligands. In addition to LRP, RAP was subsequently found to bind directly (Kounnas *et al.,* 1992; Orlando *et al.,* 1992) and inhibit other ligands from binding to megalin (Willnow *et al.,* 1992). Studies since have shown that RAP binds to other members of the LDLR family and universally inhibits ligand interactions with these receptors. Specifically, RAP exhibits high affinity binding ($K_d \sim 1$–$10$ nM) to LRP (Herz *et al.,* 1991; Iadonato *et al.,* 1993), megalin (Kounnas *et al.,* 1992; Orlando *et al.,* 1992), the VLDLR (Battey *et al.,* 1994; Simonsen *et al.,* 1994), apoER2 (Stockinger *et al.,* 1998; Sun and Soutar, 1999), and LR11 (Jacobsen *et al.,* 1996), but only weak affinity ($K_d \sim 250$ nM) to the LDLR (Medh *et al.,* 1995; Mokuno *et al.,* 1994). The potential interactions between RAP and the several newer members of the LDLR family (e.g., LRP3, LRP4, LRP5, LRP6, and

LRP1B) have not been investigated to date. In addition to members of the LDLR family, three other receptors, lipolysis-stimulated receptor (Troussard *et al.,* 1995), cubilin (Birn *et al.,* 1997), and sortilin (Petersen *et al.,* 1997; Tauris *et al.,* 1998), have also been shown to bind RAP.

The mechanism by which RAP inhibits ligand binding and/or uptake by the receptors is still not entirely clear. Binding analysis of fluorescently labeled RAP to purified LRP in solution indicated that RAP binds to two equivalent sites on each receptor molecule (Williams *et al.,* 1992). Using LRP as a target receptor expressed on hepatocytes, our early studies via saturation binding analyses have shown that LRP contains approximately five to seven times as many binding sites for RAP compared to those for tPA (Iadonato *et al.,* 1993). More recently, using truncated soluble minireceptors of LRP representing each of the putative ligand-binding domains and subdomains of LRP, we have identified at least five independent RAP-binding sites, two on each of the second and fourth, and one on the third ligand-binding domain of LRP (Bu and Rennke, 1996). In another study using surface plasmon resonance (Horn *et al.,* 1997), a high-affinity RAP-binding site was mapped within the complement-type repeats 5–7 of the second ligand-binding domain of LRP. Most recently, studies byAndersen *et al.,* (2000) have shown that the minimal functional unit in LRP for RAP binding is only two ligand-binding repeats, suggesting that each ligand-binding domain may potentially bind multiple RAP molecules. Taken together these studies suggest that each LRP molecule may contain five to seven RAP-binding sites distributed among the second, third, and fourth ligand-binding domains of LRP. These RAP-binding sites may be shared or overlap with individual ligand-binding sites on LRP, thus allowing RAP to either competitively or sterically inhibit the binding of LRP ligands. This hypothesis has been supported by reverse-competition studies, which showed that high concentrations of tPA, $\alpha_2$M* (Bu *et al.,* 1992b), or t-PA:PAI-l complexes (Horn *et al.,* 1997) can partially inhibit RAP-binding to LRP. Furthermore, the fact that RAP is capable of inhibiting ligand binding to immobilized receptor on ligand blot suggests that the flexibility of the receptor upon RAP binding is not required for blocking ligand interaction with the receptors.

An alternative model for RAP inhibition of ligand binding, which has received more experimental support recently, suggests that RAP may induce a conformational change within LRP, which precludes ligand binding (Horn *et al.,* 1997; Neels *et al.,* 1999). In support of this model, RAP possesses at least three independent receptor binding sites within each molecule (see below), capable of inducing a conformational change by interacting with multiple sites within the receptor and thereby preventing ligand binding (Bu *et al.,* 1995; Horn *et al.,* 1997). Additionally, the fact that the RAP concentration for half-maximal inhibition of LRP ligands ($K_i \sim 1$ nM) (Iadonato *et al.,* 1993; Warshawsky *et al.,* 1993) is lower than its $K_d$ value (~3 nM, Iadonato *et al.,* 1993) suggests that a complete occupation of all RAP-binding sites within each receptor is not required to achieve maximal ligand inhibition. It is important to point out that these two models of RAP inhibition of ligand binding to LRP are not mutually exclusive. In fact, it is possible that a

combination of conformational changes within LRP and competitive inhibition/steric hindrance may allow RAP to inhibit all ligand interactions with the receptor. Other members of the LDLR family likely share the same mechanisms as to how RAP antagonizes ligand interaction with the receptors.

Although it has been demonstrated that multiple RAP-binding sites exist within LRP, it is not clear whether RAP uses the same binding epitope for all its binding sites on the receptor. Indirect evidence suggests that these sites are probably not all identical. For example, using recombinant RAP fragments that represent different regions of RAP, we found that these truncated RAP fragments bind differentially to various domains of LRP (Warshawsky *et al.,* 1994, 1995). In addition, our more recent studies, utilizing LRP minireceptors that represent individual ligand-binding domains and truncated recombinant RAP, showed that while the carboxyl-terminal region of RAP possesses high affinities to each of the three ligand-binding domains on LRP (domains II, III, and IV), the amino-terminal and central regions of RAP exhibit only low affinity to the second and the fourth ligand-binding domains of LRP, respectively (Obermoeller *et al.,* 1997). Finally, it was found that the binding of at least two of the LRP ligands, $\alpha_2$M* and tPA, is differentially inhibited by RAP fragments (Warshawsky *et al.,* 1993, 1994, 1995). These multiple LRP-binding sites within RAP and multiple RAP-binding sites within LRP may contribute to the formation of large molecular size complexes during receptor folding and trafficking (see below) and in the inhibition of ligand binding to the receptors. Obviously, precise definition of RAP–LRP interactions at the different sites requires future investigation.

It is intriguing to compare some of the structural and functional properties between RAP and apoE, which is one of the ligands for LRP. For example, these proteins are similar in size and both contain highly ordered helical structures that include antiparallel helix bundles (Nielsen *et al.,* 1997; Rall *et al.,* 1998; Wetterau *et al.,* 1998; Wilson *et al.,* 1991). These helix bundle domains are resistant to proteolysis and have guanidine HC1 denaturation transitions at about 2.4 M (Rall *et al.,* 1998; Wetterau *et al.,* 1988). In addition, Strickland *et al.* (1991) have observed sequence and potential structural similarities between the carboxyl-terminal region of RAP and the amino-terminal region of apoE. These regions within RAP and apoE also share functional similarities as well; both bind to members of the LDLR family and to heparin (Obermoeller *et al.,* 1997; Orlando and Farquhar, 1994; Warshawsky *et al.,* 1993; Weisgraber *et al.,* 1986; Wilson *et al.,* 1991). Since the binding of apoE to the LDLR is mediated through a cluster of basic amino acid residues within apoE amino acids 136–150 (Innerarity *et al.,* 1983; Weisgraber *et al.,* 1983; Wilson *et al.,* 1991), it is tempting to suggest that similar residues within RAP are also important for receptor binding. In support of this hypothesis, studies by Warshawsky *et al.* (1993, 1995), using LRP as a model receptor, have identified clusters of highly charged amino acid residues within RAP that are required for interaction with LRP. Similar interactions have been suggested for interactions between other LRP ligands and the receptor. For example, a cryptic high-affinity binding site within PAI-l has been shown to be exposed

upon complex formation with tPA and is responsible for high-affinity receptor and heparin binding (Horn *et al.,* 1998; Stefansson *et al.,* 1998). The exact residues within RAP that participate in receptor binding require future mutagenesis analysis and structural examination. In addition to receptor binding, basis amino acid residues within the carboxyl-terminus of RAP have been speculated to mediate RAP binding to heparin (Orlando and Farquhar, 1994; Warshawsky *et al.,* 1993). It will be of interest to investigate whether similar basic residues within RAP participate in both receptor and heparin binding. As many of the LRP ligands are also heparin-binding proteins, it is possible that binding to both receptor and heparin is mediated by a similar mechanism, i.e., electrostatic interaction. Finally, Hiesberger *et al.* (1996) found that an antibody generated against RAP strongly cross-reacts with members of the LDLR family, suggesting that RAP and these receptors may share a common epitope. The biochemical significance of this latter observation is not clear at present.

Within the receptor sequences, it has been hypothesized that several conserved acidic amino acid residues within the LDLR repeats mediate its interaction with apoE (Goldstein *et al.,* 1985; Mahley, 1988; Wilson *et al.,* 1991), although recent crystal structural analysis of apoE indicated that some of these residues in fact participate in the formation of a $Ca^{2+}$ cage (Fass *et al.,* 1997). Thus, it is likely that certain acidic residues within the ligand-binding repeats of the LDLR family mediate binding of both apoE and RAP. In support of this hypothesis, a study byAndersen *et al.* (2000) has shown that a conserved acidic residue that is present in several ligand-binding repeats within LRP, and is not part of the $Ca^{2+}$ cage, is required for high affinity binding to RAP. Following the alignment of the ligand-binding repeats from several receptors of the LDLR family, the authors concluded that the presence or absence of this residue within these repeats may explain why different receptor/ligand-binding domains exhibit differential affinity for RAP.

## C. RAP as a Special Reagent in the Study of Receptors in the LDLR Family

The discovery of RAP's ability to inhibit ligand binding to LRP, and to other members of the LDLR family, prompted the use of recombinant RAP as an antagonist in the study of these receptors. This application of RAP was highlighted in the identification of novel ligands for LRP (Bu *et al.,* 1992a, 1992b; Kounnas *et al.,* 1992; Nykjaer *et al.,* 1992; Orth *et al.,* 1992; Willnow *et al.,* 1992). Thus, one of the standards in defining a new ligand for LRP is its inhibition by RAP. However, since RAP is an antagonist for all members of the LDLR family, inhibition of ligand binding and/or uptake by RAP indicates only that some member of the LDLR family is likely involved. Thus, other experimental approaches and reagents are required for defining the participation of a specific receptor. For example, antibodies specific to individual receptors in the LDLR family (Holtzman *et al.,* 1995; Stockinger *et al.,*

1998), or cell lines that either lack or express specific receptors (FitzGerald *et al.,* 1995; Willnow and Herz, 1994), have been utilized to define the involvement of a specific receptor. On the other hand, if a certain ligand binds only to a single receptor within the LDLR family (e.g., $\alpha_2$M* to LRP), inhibition by RAP would be sufficient to conclude its involvement.

Another use of RAP in the study of receptor biology is in the examination of potential *in vivo* functions of receptors within the LDLR family. The first example of such an approach showed that infusion of recombinant RAP in rats prolonged the plasma half-life of the LRP ligand tPA (Warshawsky *et al.,* 1993). Subsequently, using an adenoviral gene-delivery system, Willnow *et al.* (1994) reported the transient overexpression of RAP in the liver of mice. RAP, accumulated in plasma of these virally infected mice, was sufficient to inhibit LRP-mediated clearance of ligands by hepatic cells. When combined with LDLR-knockout mice, this viral delivery system allowed the differentiation of effects *in vivo* lipoprotein metabolism attributable to the LDLR and to LRP (Willnow *et al.,* 1994). Using the same adenoviral delivery system, Narita *et al.* (1995) have shown that two receptor systems, LRP and the mannose receptor, are involved in the plasma clearance of tPA. Similarly, it was found that both LRP and cell surface heparan sulfate proteoglycan (HSPG) are functional in the plasma removal of TFPI (Narita *et al.,* 1995). Finally, a recent study with adenoviral expressed RAP has identified an extrahepatic mechanism for the metabolism of triglyceride-rich lipoproteins that is independent of the LDLR and LRP (van Vlijmen *et al.,* 1999). Exogenous RAP will likely also be used to study the functions of LDLR members within the CNS, either by administering recombinant RAP into the cerebral spinal fluid (CSF) or an infection of RAP-expressing virus with subsequent expression in brain parenchyma. In addition to these *in vivo* analyses, recombinant RAP may also be used in certain semi-*in vivo* systems. For example, we have recently utilized RAP to inhibit LRP function in long-term potentiation with mouse hippocampal slices (Zhuo *et al.,* 2000). Thus, the ability of RAP as a universal antagonist for LDLR family members provides a unique tool for the biological study of these receptors. This is particularly important for studying certain members of the LDLR family (e.g., LRP and megalin) in adult mice, since deletion of their genes is lethal during mouse development (Herz *et al.,* 1992; Willnow *et al.,* 1996).

## IV. RAP Safeguards Receptors during Their Exocytic Trafficking

### A. Intracellular Localization of RAP

The molecular cloning of RAP focused speculation as to its physiological function (strickland *et al.,* 1991). The fact that RAP contains a signal peptide suggests that

this protein may be a secreted protein, a feature that could allow regulation of the receptor on the cell surface by RAP. However, pulse-chase biosynthetic analyses of RAP did not detect secretion of this protein in the extracellular media, although direct cell surface radio labeling did demonstrate this protein on the cell surface along with LRP subunits (Strickland *et al.,* 1991). The first intracellular localization of RAP was described by Orlando *et al.* (1992) when examining the localization of megalin and RAP in kidney proximal tubules. Using immunofluorescent and immunoelectron microscopy, they showed that RAP was found intracellularly within the ER. The ER localization of RAP in kidney proximal tubules was also reported byAbbate *et al.* (1993). The intracellular distribution of RAP was later quantitatively defined using colloidal gold immunoelectron microscopy with human glioblastoma U87 cells, which express both abundant RAP and LRP (Bu *et al.,* 1994a). RAP was localized most abundantly within the ER (70%) and early Golgi compartments (24%) with little found at the cell surface or in the endosomes. This distribution pattern of RAP is similar to several ER resident proteins, and suggested intracellular retention/retrieval of this protein within the early secretory pathway. Indeed, molecular and cellular examination of RAP indicated that the HNEL sequence at the carboxyl-terminus of RAP is both required and sufficient for its retrieval from the Golgi to the ER (Bu *et al.,* 1995, 1997), an intracellular trafficking event mediated by the KDEL receptors/ERD2 proteins (Bu *et al.,* 1997; Rothman and Orci, 1992). Removal of this signal from RAP results in secretion and redistribution of RAP along the secretory pathway, similar to that seen for normally secreted proteins (Bu *et al.,* 1995). An electron microscopy immunogold staining of RAP and RAPΔHNEL illustrating the differential localization of these proteins is shown in Fig. 2. Thus, RAP is an ER-resident protein with its carboxyl-terminal HNEL sequence serving as the ER-retention signal.

## B. Binding of RAP to Receptors Prevents Premature Binding of Ligands

Although recombinant RAP was used extensively as an antagonist to inhibit ligand binding and/or uptake at the cell surface, the observation that RAP is distributed within the ER and early Golgi compartments suggested that the normal physiological function of RAP is likely intracellular. Two studies of the biosynthesis of the Heymann nephritis antigenic complex demonstrated an association of the two component proteins, megalin and RAP, within the early secretory pathway in the rat kidney (Biemesderfer *et al.,* 1993; Orlando *et al.,* 1992). Such an association was also observed in L2 rat yolk sac cells (Lundstrom *et al.,* 1993) and in differentiated F9 embryonic cells (Czekay *et al.,* 1995). In addition, a direct interaction between RAP and LRP within intact cells was demonstrated in U87 cells using a membrane-permeable chemical cross-linker DSP (Bu *et al.,* 1995). Pulse-chase analysis reveals that RAP–LRP association is transient early in the

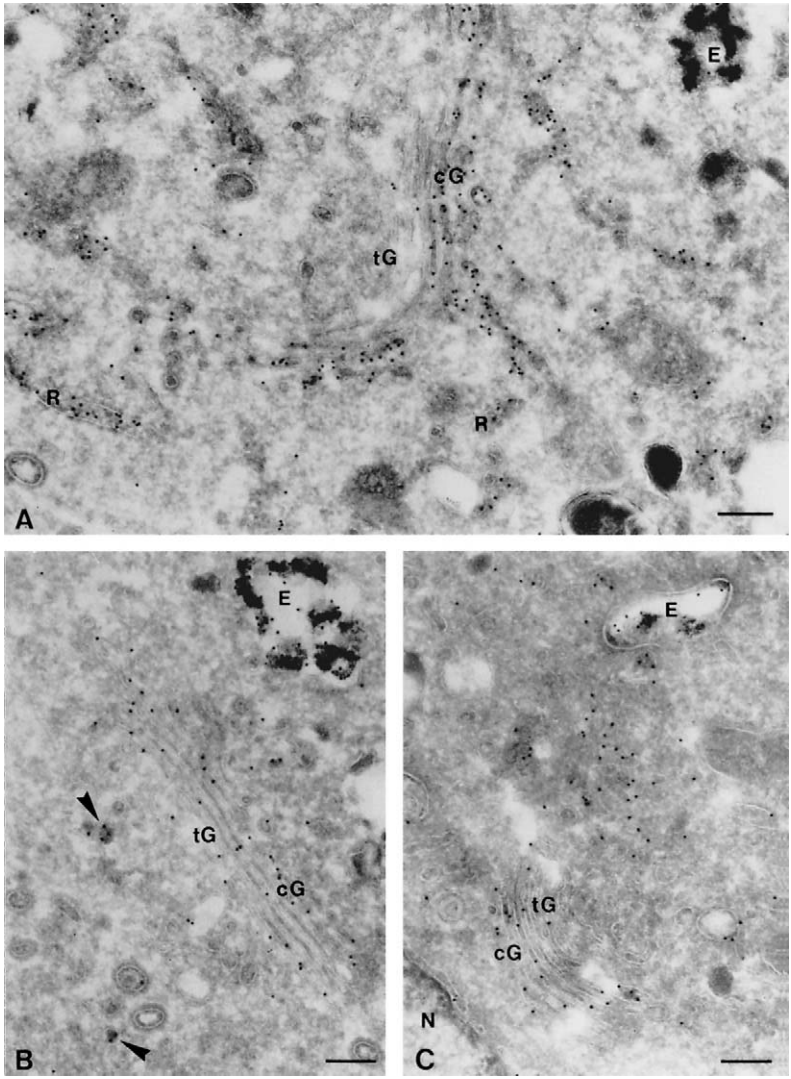
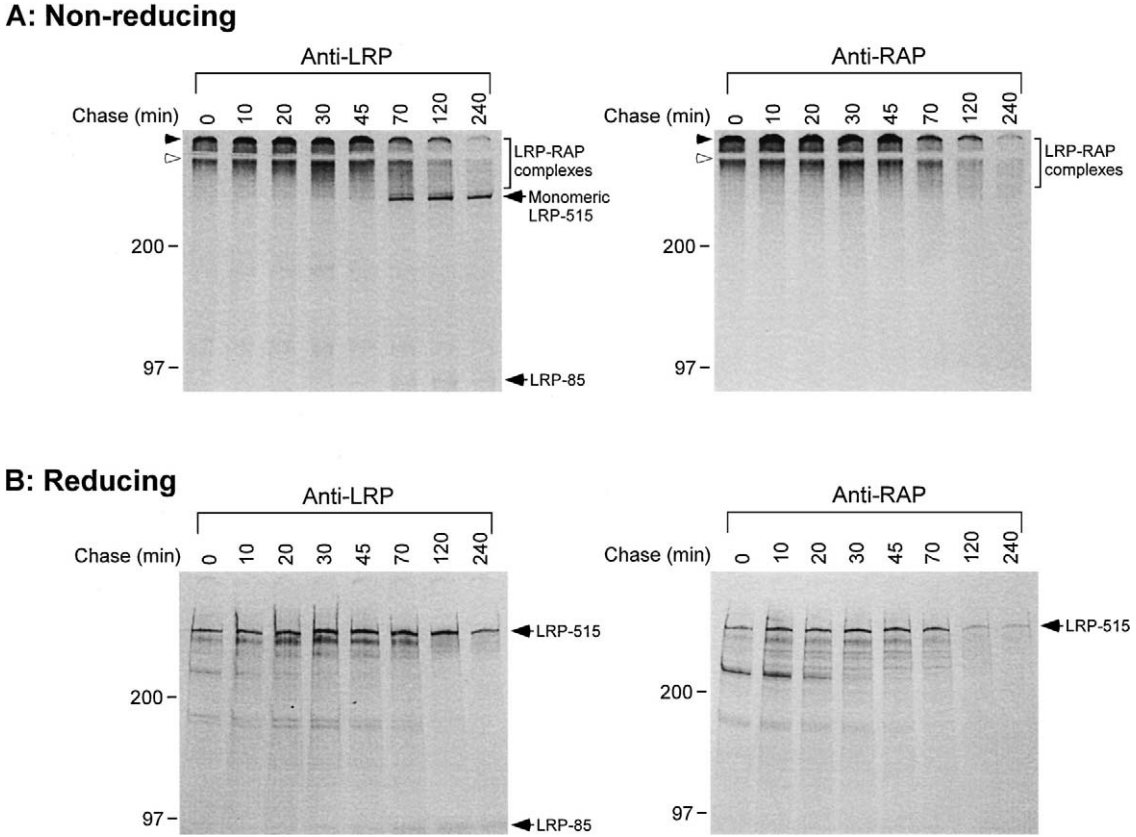



FIG. 2 Immunogold electron microscopy localization of RAP and RAPΔHNEL. Immunoelectron micrographs of ultrathin cryosections from RAP- or RAPΔHNEL-stably transfected U87 cells labeled with 10-nm gold particles for the demonstration of RAP (A) and RAPΔHNEL (B and C). Prior to fixation, the cells were allowed to endocytose 5-nm BSA–gold particles for 1 h. Bars represent 200 nm. (A) RAP-transfected cells showing RAP present in the rough ER (R) and in *cis*-Golgi elements (cG). The *trans*-Golgi elements (tG) are largely devoid of detectable RAP. RAP was occasionally found in endosomes marked with endocytosed BSA–gold particles (E). (B and C) RAPΔHNEL-transfected cells showing RAPΔHNEL present throughout the Golgi stacks, in endosomes (E) containing BSA–gold particles and in electron-dense vesicles (arrowhead) in the *trans*-Golgi area. N, nucleus. (Reproduced by permission from Oxford University Press, from Bu, G., Geuze, H. J., Strous, G. J., and Schwartz, A. L. 39 kDa receptor–associated protein is an ER resident protein and molecular chaperone for LDL receptor–related protein. *EMBO Journal,* 1995;14:2269–2280.)

secretory pathway and coincides with the formation of high-molecular-weight RAP–LRP complexes and reduced ligand-binding activity of the receptor. Results from a representative pulse-chase experiment depicting RAP–LRP interaction following receptor biosynthesis is shown in Fig. 3. In this experiment, U87 cells were pulse-labeled with [$^{35}$S]cysteine for 20 min, followed with chase in the presence of unlabeled cysteine for the indicated times. After each chase period, interacting proteins were cross-linked with the membrane-permeable, thio-cleavable, cross-linker DSP. Cell lysates were then immunoprecipitated with either anti-LRP or anti-RAP antibodies and analyzed via SDS–PAGE (5% acrylamide) under either nonreducing (Fig. 3A) or reducing (Fig. 3B) conditions. Since the chemical cross-linking was performed on intact cells, these results should reflect true interaction between cross-linked proteins. As seen in the figure, with anti-LRP antibody and non reducing SDS–PAGE, LRP exists exclusively as high-molecular-size complexes early in the chase. RAP is also a component of these complexes since anti-RAP antibody immunoprecipitated radiolabled proteins with an identical pattern seen with anti-LRP antibody. Monomeric LRP started to appear after ~70 min of chase, with a concomitant decrease of high-molecular-size species (Fig. 3A, anti-LRP). However, the monomeric LRP is not immunoprecipitated by anti-RAP antibody (Fig. 3A, anti-RAP). These results indicate that RAP associates with LRP following the biosynthesis of the receptor, and traffics together with LRP in large-molecular-size complexes during the early stages of exocytic trafficking. LRP and RAP are the major components of the cross-linked complexes since only LRP and perhaps some of the intermediates of LRP found during synthesis are seen under reducing conditions (Fig. 3B). RAP is not labeled in this experiment since its sequence does not contain cysteine residues (Strickland *et al.,* 1991). RAP appears to dissociate from LRP when the complexes dissolve into monomeric receptors (~70 min following the chase). Our previous studies have also shown that this dissociation of RAP from LRP during the receptor's exocytic trafficking correlates with an increase in LRP ligand-binding activity (Bu *et al.,* 1995). Thus, the ability of RAP to antagonize ligand binding to the receptor is utilized during biogenesis as a mechanism to safeguard the receptor during its exocytic trafficking. This is an important event as all LRP ligands are secretory/membrane proteins and share the same exocytic pathway as does the receptor.

---

FIG. 3 Kinetics of RAP association with LRP. U87 cells were pulse-labeled with [$^{35}$S]cysteine for 20 min and chased for increasing periods of time as indicated. After each chase, cellular proteins were cross-linked with membrane-permeable cross-linker DSP. Cell lysates were then immunoprecipitated with either anti-LRP antibody or anti-RAP antibody and analyzed via 5% SDS–PAGE under either nonreducing (A) or reducing (B) conditions. The positions of LRP-515 and LRP-85 subunits, as well as LRP–RAP complexes in nonreducing gels, are indicated. The top of the stacking gels is marked with a closed arrowhead, whereas the top of separating gels is labeled with an open arrowhead. The 200- and 97-kDa molecular size markers are also shown. Note the appearance of monomeric LRP in nonreducing gel (A) is seen only with anti-LRP, but not with anti-RAP antibodies.


A: Non-reducing
Anti-LRP
Chase (min)
0
10
20
30
45
70
120
240
LRP-RAP complexes
Monomeric LRP-515
200
97
LRP-85
Anti-RAP
Chase (min)
0
10
20
30
45
70
120
240
LRP-RAP complexes
200
97
B: Reducing
Anti-LRP
Chase (min)
0
10
20
30
45
70
120
240
LRP-515
200
97
LRP-85
Anti-RAP
Chase (min)
0
10
20
30
45
70
120
240
LRP-515
200
97

This chaperone/escort function of RAP is also supported by several other studies. Most notable, the expression of LRP, megalin, and the VLDLR was found to be markedly impaired in tissues of RAP-knockout mice (Willnow *et al.,* 1995, 1996). In case of LRP, an approximately 75% reduction at both protein and functional level was observed in the liver and brain in RAP-knockout mice compared to wild-type controls. On the other hand, a relative increase of the ER precursor forms for megalin and the VLDLR was found in the kidney and the heart, respectively, in RAP-knockout mice. These differences between LRP and megalin/VLDLR may reflect a difference in the degradation of misfolded protein within the ER. Although, the initial observation of RAP-knockout mice suggested a normal phenotype (Willnow *et al.,* 1995), a more recent study has shown that these mice are cognitively impaired as assessed via the Morris water maze test compared to controls. In addition, they showed a significant decrease in number of somatostatin-expressing neurons compared to that seen in controls (Van Uden *et al.,* 1999). This new study clearly suggests an important role of LRP, and/or other receptors within the LDLR family (Herz *et al.,* 2000; Kim *et al.,* 1996; Stockinger *et al.,* 1998; Zheng *et al.,* 1994), in neuronal development and cognitive function (also see Gliemann, 1998; Willnow, 1999).

Biochemical and cellular studies have shown that a significant amount of LRP is aggregated and retained within the ER in hepatocytes derived from RAP-knockout mice (Willnow *et al.,* 1996). Such ER retention was postulated to be ligand-induced and was abrogated by the coexpression of RAP (Willnow *et al.,* 1996). Without RAP, LRP may be subject to faster turnover due to abnormal aggregation resulting from premature interactions with its ligands and/or improper folding (see below). These results together provide direct evidence that RAP regulates LRP's ligand-binding activity within the secretory pathway and suggest a role for RAP in preventing premature interaction of LRP with its ligands during the receptor's exocytic trafficking. A similar chaperone/escort function of RAP is likely to apply to other members of the LDLR family. A model depicting the function of RAP during the biogenesis of receptors of the LDLR family within the early secretory pathway is shown in Fig. 4.

The function of RAP in regulating the ligand-binding activity during receptor exocytic trafficking shares similarities with those of other chaperone/escort proteins. For example, Braks and Martens (1994) showed that the neuronal endocrine polypeptide 7B2 prevents the premature activation of the prohormone convertase PC2 within the secretory pathway. Similarly, the heat-shock protein 90 (hsp90) was found to regulate the glucocorticoid receptor activity (Pratt, 1993). Finally, the escort function of RAP resembles that of the invariant chain in regulating the peptide binding activity of MHC class II molecules (Sant and Miller, 1994). In this latter case, binding of the invariant chain to MHC class II molecules prevents premature peptide binding during its biosynthesis and trafficking within the early secretory pathway.

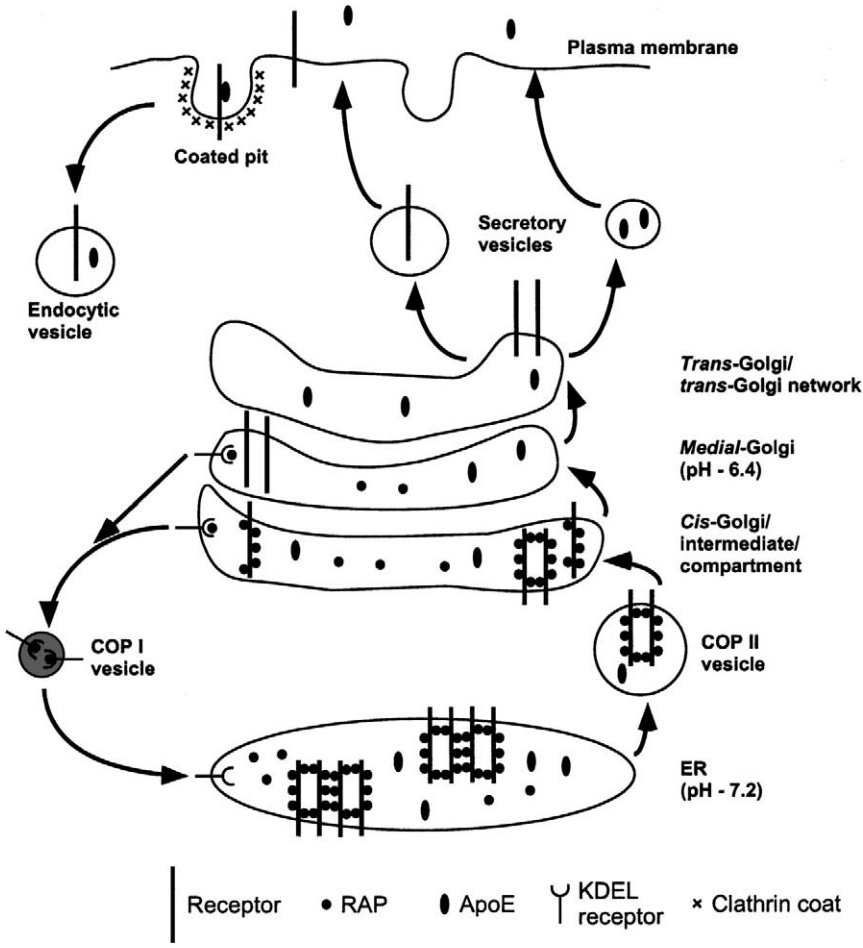



FIG. 4 Schematic model depicting how RAP functions as a chaperone/escort protein for LDLR family members. During the biosynthesis of the receptors, RAP associates with these receptors and initiates the formation of multimolecular RAP–receptor complexes. Within these complexes, RAP assists in the proper folding of the receptors. During their subsequent trafficking, RAP remains associated with the receptors to prevent premature interactions of ligands (apoE is included as a representative ligand) with the receptors. Following their trafficking together to the *medial*-Golgi compartments, RAP dissociates from these receptors as a result of the low pH. The retrieval receptors (KDEL receptors/ERD2 proteins) shuttle RAP via COP I vesicle back to the ER where neutral pH unloads RAP from its retrieval receptor. The low pH within the *medial*-Golgi, which facilitates RAP dissociation, may also impede the binding of other ligands to the receptors within the terminal portions of the secretory pathway. This facilitates the coordinate secretion of ligands and delivery of ligand-free receptors to the cell surface.

## C. RAP Recycles between ER and Golgi by Default

How does RAP dissociate from the receptors and return to the ER following its trafficking to the Golgi together with the receptors? Our *in vitro* studies have shown that the ability of RAP to associate with LRP drops significantly at pH $\leq$ 6.6, a condition found within the *cis*- and *medial*-Golgi compartments (Bu *et al.,* 1995). In addition, we found that the affinity of radiolabeled RAP for LRP decreases significantly as pH decreases below neutral. Thus, under physiological conditions the change in pH to mildly acidic conditions within the *cis*- and the *medial*-Golgi compartments is likely the cause of dissociation of RAP from the receptors. The decreased affinity of RAP for LRP at lower pH may reflect the nature of ligand–receptor interaction and may share similarity with the mechanism as to how ligands dissociate from the receptors within the early/sorting endosomes following endocytosis. The dissociated RAP within the *cis*- or *medial*-Golgi compartments then binds to the KDEL receptors (Bu *et al.,* 1997), which prefer ligand binding under acidic conditions (Wilson *et al.,* 1993). Following the transport of RAP from the Golgi compartments back to the ER via COPI-mediated vesicular transport (Scales *et al.,* 2000), RAP dissociates from the KDEL receptors due to the neutral pH environment within the ER. This recycling of RAP between the ER and the Golgi compartments allows RAP to be reutilized as a molecular chaperone for members of the LDLR family many times during its own lifetime.

Although the recycling of RAP from Golgi to the ER constitutes a default pathway for RAP trafficking, the KDEL receptor-mediated retrieval system can be saturable. For example, overexpression of RAP via the adenoviral system (see above) results in saturation of the retrieval system and the secretion of RAP into the extracellular media (Willnow *et al.,* 1994). In addition, when either a KDEL- or a HNEL-containing protein is overexpressed in stably transfected cells, these proteins compete for the KDEL receptors, and result in the secretion of endogenous RAP (Bu *et al.,* 1997). Thus, it is possible that RAP could be secreted when either its expression or the expression of other KDEL/HNEL-containing proteins are upregulated under certain physiological or pathophysiological conditions. In fact, several studies have detected cell surface localization of RAP. For example, immuno-staining analyses have found RAP on the apical surface of kidney proximal tubule cells (Orlando *et al.,* 1992; Pelham, 1990) and on the surface of rat yolk sac carcinoma cells (Orlando and Farquhar, 1993). In addition, RAP was detected via cell surface iodination on the surface of gingival fibroblasts (Strickland *et al.,* 1991). Finally, using flow cytometric analysis, Li *et al.* found significant amounts of RAP on the cell surface of two melanoma cell lines (Li *et al.,* 1998). Since RAP is a potent antagonist for all ligand interactions with members of the LDLR family, it may be important for cells to sequester those RAP molecules that have escaped the retrieval system. The fact that RAP is a heparin-binding protein (Moestrup and Gliemann, 1991; Orlando and Farquhar, 1994; Warshawsky *et al.,* 1993) suggests that the majority of secreted RAP likely bind to the large pool of cell

surface HSPG. Such an interaction between RAP and cell surface HSPG provides a mechanism by which secreted RAP molecules are rapidly and efficiently trapped and subsequently delivered to members of the LDLR family for degradation via the endocytic pathway (Czekay *et al.,* 1997). This type of "safety" mechanism can prevent potential nonproductive paracrine functions of RAP (e.g., inhibition of ligand binding to members of the LDLR family on the cell surface, either in systemic or micro-extracellular environments).

One unresolved question is whether the distribution of RAP in the Golgi (Bu *et al.,* 1994) is solely due to its association with the receptors, or whether it can be transported alone to that compartment via a default mechanism. Our recent studies have shown that the endoglycosidase H-resistant form of RAP is detected at significantly higher levels in cells overexpressing LRP minireceptors (Obermoeller-McCormick *et al.,* unpublished results). These results suggest that LRP facilitates, if not essential for, RAP trafficking to the Golgi compartments.

## V. RAP Is Also a Folding Chaperone for Members of the LDLR Family

### A. RAP Is a Folding Chaperone

The complex structures of the LDLR family members, largely due to the extensive intradomain disulfide bonds, present a challenging task for their proper folding during biosynthesis. This process may well be assisted by both classical chaperones (e.g., BiP, protein disulfide isomerase, and calnexin) and specialized chaperone(s) within the ER. Since RAP is the only known specialized chaperone that interacts with these receptors, it is possible that RAP may be directly involved in receptor folding. To test this hypothesis, we generated anchor-free, soluble minireceptors that represent each of the four putative ligand-binding domains of LRP (termed sLRP1, -2, -3, and -4, corresponding to the clusters with 2, 8, 10, and 11 ligand-binding repeats, respectively). When these sLRPs are expressed via cell transfection, we found that, in the absence of RAP coexpression, sLRP2, sLRP3, and sLRP4 are oligomerized and are retained within the ER (Bu and Rennke, 1996). These oligomers of the sLRPs result from the formation of intermolecular disulfide bonds during misfolding, since they were reduced to monomers when analyzed under reducing conditions. When RAP cDNA was cotransfected with that of sLRP2, -3, or -4, each of these sLRPs was folded correctly and secreted, with few or no aggregated oligomers. These results suggest that interactions between RAP and multiple sites on LRP may be important for proper folding of LRP by ensuring the formation of proper intradomain, but not intermolecular, disulfide bonds. In addition to soluble LRP minireceptors, the role of RAP as a folding chaperone has also been demonstrated

with membrane-containing LRP minireceptors (mLRPs, see Obermoeller *et al.,* 1998). In that study, it was also shown that $Ca^{2+}$ ions are essential for proper folding of mLRPs as well as for endogenous LRP, likely by initiating a nucleation process during folding. Furthermore, it was found that the functions of RAP and $Ca^{2+}$ ions during LRP folding are independent (Obermoeller *et al.,* 1998).

The role of RAP as a folding chaperone for lipoprotein receptors has since been extended to other members of the LDLR family. For example, our recent studies have shown that proper folding and trafficking of both soluble and membrane-containing forms of the VLDLR is promoted by the coexpression of RAP (Savonen *et al.,* 1999). Similarly, studies by Sato *et al.* (1999) have shown that coexpression of RAP facilitated the secretion of soluble VLDLR. Thus, RAP likely promotes the folding of all members of the LDLR family that bind to this protein, by assisting the formation of proper disulfide bonds. An exception to that may be the LDLR, which exhibits much lower affinity to RAP compared to other receptors (Medh *et al.,* 1995). In this regard, studies by Sato (1999) have shown that, while the folding process of soluble LRP and the VLDLR is facilitated by RAP coexpression, folding and secretion of the LDLR is not. Such results are consistent with the observation that while processing of LRP, megalin, and the VLDLR is variably impaired in RAP-knockout mice, the functional expression of the LDLR is unaffected (Willnow *et al.,* 1995, 1996). It is possible that the folding process of the LDLR requires a separate chaperone that functions similarly to RAP. Alternatively, folding and biogenesis of the LDLR may be facilitated by coexpression of RAP only under certain physiological or pathophysiological conditions, due either to mutations of the LDLR that influence its folding (e.g., Class II LDLR mutants; see Brown and Goldstein, 1986) or to overexpression of this receptor. Finally, it is important to point out that RAP may also play a role in the biogenesis of other proteins since previous studies have identified several RAP-interacting proteins that do not belong to the LDLR family (see above) (Birn *et al.,* 1997; Petersen *et al.,* 1997; Tauris *et al.,* 1998; Troussard *et al.,* 1995).

## B. The Function of RAP in Receptor Folding and Trafficking Are Independent

The dual functions of RAP in receptor folding and trafficking provide a novel model by which a single chaperone/escort protein performs two independent tasks. However, whether these two functions in the biogenesis of LDLR family members are related or are independent is not clear. For example, the function of RAP in receptor folding may depend on its ability to prevent ligand interaction with the receptor during the folding process. In support of this hypothesis, Willnow *et al.* (1996) have shown that overexpression of a LRP ligand, apoE, results in an accumulation of LRP within the ER, an event that can be prevented by a coexpression of RAP. However, whether the ER-retention of LRP in apoE-expressing cells was due to misfolding of LRP was not clear. A similar study was performed using primary cultured

hippocampal neurons derived from RAP-knockout mice (Umans *et al.,* 1999). Herein, it was found that LRP exhibited slower maturation and corresponding accumulation within the ER in these RAP-deficient neurons. In addition, LRP was redistributed over the cell body at the expense of the dendrites. However, it was noted that in these neurons neither the added expression of apoE nor that of APP influenced the maturation of LRP, in either the presence or the absence of RAP (Umans *et al.,* 1999). These results suggest that although RAP serves as a chaperone/escort protein for LRP, it is not required for the coexpression of LRP with its ligands.

Another approach that may allow for the differentiation of RAP functions in receptor folding and in safeguarding the receptors during their trafficking is to utilize domain constructs of RAP and/or the receptors. For example, using recombinant RAP constructs that represent each of the three internal repeats (see below), we found that only the carboxyl-terminal repeat of RAP was able to promote the folding and subsequent secretion of soluble LRP minireceptors (Obermoeller *et al.,* 1997). However, when the ability of each RAP repeat to inhibit ligand interaction with LRP was examined, differential effects were observed for different LRP ligands. Most noticeable, both the amino-terminal and central repeats, but not the carboxyl-terminal repeat, of RAP inhibited the interaction of $\alpha_2$M* with LRP. On the other hand, only the carboxyl-terminal repeat, but not the amino-terminal and central repeats, of RAP inhibited tPA binding to LRP (Obermoeller *et al.,* 1997). It appears that high affinity interaction of RAP with LRP, which relies on the presence of the carboxyl-terminal repeat of RAP, is required for its function in receptor folding. On the other hand, the ability of RAP to inhibit individual ligand interaction with the receptors may reside within different epitopes within RAP. These differential functions of the RAP repeats suggest that the roles of RAP in the folding of LRP and in the prevention of premature interaction of ligand with the receptor are independent.

Further dissection of RAP function in receptor folding and trafficking may rely on an *in vitro* refolding system, in which RAP and/or receptor ligands can be added to or deleted from the system during receptor folding. This type of study should be possible since successful *in vitro* refolding of RAP-binding receptors has been reported for the LDLR (Simmons *et al.,* 1997) and LRP fragments (Andersen *et al.,* 2000; Vash *et al.,* 1998). If RAP can promote the folding of these receptors in the absence of added receptor ligands, it would suggest that this function of RAP in receptor folding is independent from its function in the prevention of ligand binding to the receptors.

## VI. Internal Sequence Triplication and Structure of RAP

Early studies byWarshawsky *et al.* (1993, 1994) have shown that the amino-terminal and the carboxyl-terminal domains of RAP can interact with LRP independently, suggesting that more than one receptor binding epitope exists within RAP.

Subsequently, an internal sequence triplication of RAP was noted as high sequence homology was observed when RAP is divided into three approximately equal parts, namely residues 1–100, 101–200, and 300–323 (named repeats 1, 2, 3; or amino-terminal, central, carboxyl-terminal repeats, respectively; see Bu *et al.,* 1995; Warshawsky *et al.,* 1995, Obermoeller *et al.,* 1997; and also Fig. 5). A detailed homology analysis showed that both repeat 1 and repeat 2 have high homology with repeat 3 (46.4 and 45.5% similarity, respectively), whereas repeat 1 and repeat 2 have relatively low homology (38.9% similarity; see Obermoeller *et al.,* 1997). Thus, it was speculated that if the three repeats of RAP were derived from the same ancestral sequence, repeat 1 and repeat 2 may have been derived from repeat 3 separately during evolution. This internal triplication of RAP has been confirmed by studies from other investigators. For example, studies by Ellgaard *et al.* (1997) have confirmed the presence of the three repeats within RAP, but proposed slightly shifted boundaries for these repeats (i.e., residues 18–112 for repeat 1, 113–218 for repeat 2, and 219–323 for repeat 3). In addition, using differential scanning calorimetry, circular dichroism, and fluorescence spectroscopy, Medved *et al.* (1999) have proposed four independent folded domains within RAP (residues 1–92, 93–163, 164–216, and 217–323), with domains 2 and 3 together equivalent to the second repeat defined previously. Finally, the presence of the three repeats within RAP was confirmed by biochemical analyses of RAP. It was found that clusters of protease-sensitive sites are present between repeat 1 and repeat 2, as well as between repeat 2 and repeat 3, whereas these sites are virtually absent within repeats 1 and 3 (Rall *et al.,* 1998), suggesting the stability and structural independence of these repeats. Repeat 2, on the other hand, contains several internal protease-sensitive sites, suggesting that the structure of this repeat is much less stable than those of repeats 1 and 3.

What is the functional significance of sequence triplication within RAP? When recombinant RAP fragments representing each of the three repeats were tested for receptor interaction, each of the three repeats was found to bind independently to LRP, with repeat 3 exhibiting highest binding (Bu *et al.,* 1995; Bu and Rennke,

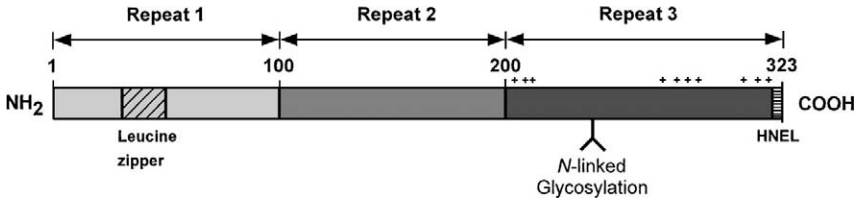



FIG. 5 Structural diagram of major features of human RAP. Human RAP (323 amino acid residues) is shown in its three internal repeat structures. The leucine zipper motif, the putative N-linked glycosylation sites, and the ER-retention signal (HNEL) at the carboxyl-terminus are marked. Three clusters of basic amino acid residues within repeat 3 (residues 203–206, 281–208, and 314–319), which are likely involved in receptor and/or heparin binding, are labeled.

1996; Obermoeller *et al.,* 1997). These results suggest that each RAP molecule is capable of interacting with more than one LRP molecule simultaneously. This potential mechanism may provide a means for RAP and LRP to form large molecular complexes during receptor folding and trafficking (see Fig. 3). Alternatively, RAP may use these multiple epitopes to bind different sites within a single LRP molecule, a mechanism that may contribute to inhibition of ligand binding by RAP. In this regard, it was shown that different repeats of RAP exhibit differential binding to different ligand-binding domains of LRP (Bu and Rennke, 1996; Obermoeller *et al.,* 1997).

The differential functions of the RAP repeats may also explain their roles and appearance during evolution. Since repeat 3 of RAP is capable of promoting the folding of LRP and inhibiting most ligand interactions with the receptor (Obermoeller *et al.,* 1997), it is likely that this repeat is the ancestral region for the whole molecule, a hypothesis that is consistent with the indications from sequence alignment (see above). However, the fact that interaction of some of the LRP ligands (e.g., $\alpha_2M^*$) is not inhibited by repeat 3 of RAP, but is by repeats 1 or 2 (Obermoeller *et al.,* 1997), suggests a need for other regions of the molecule. In this regard, it is interesting to note that both LRP (Yochem and Greenwald, 1993) and RAP (Center, 1998) are present in organisms as primitive as the nematode *Caenorhabditis elegans*. Thus, in future studies it will be of interest to examine the appearance of LRP ligands during evolution. It is possible that the appearance of some of the systemic LRP ligands was later during evolution when compared to LRP and RAP. For example, the earliest organism in which $\alpha_2M$ has been described is the horseshoe crab (Armstrong *et al.,* 1991). If most of the LRP ligands are absent in the nematode, the primary function of RAP may be to aid in receptor folding. Thus, the role of RAP in inhibiting ligand interaction with the receptor may have evolved only after the expression of LRP became high in certain tissues (e.g., liver and brain) of higher organisms and with the appearance of the diverse array of LRP ligands. The *C. elegans* homolog of RAP shares high sequence homology with human RAP, particularly in the portion where repeat 2 and repeat 3 of human RAP share the highest homology (Obermoeller *et al.,* 1997). However, no ER-retention signal is present at the carboxyl-terminus of this sequence, suggesting that the ER-retention and recycling of RAP may also have been acquired during evolution.

The solution structure of the amino-terminal repeat of RAP has been revealed by NMR spectroscopy (Nielsen *et al.,* 1997). It was found that this repeat of RAP consists mainly of three up–down–up antiparallel helices (residues 23–34, 39–65, 73–88). In addition, by examining protease sensitivity and guanidine–HCI denaturation properties of RAP and its fragments, Rall *et al.* (1998) have proposed a structural interaction between repeat 1 and repeat 2 of RAP. A similar interaction was also suggested by an independent study (Medved *et al.,* 1999). A schematic three-dimensional model of RAP derived from both structural analyses, as well as secondary structure prediction, is shown in Fig. 6 (Rall *et al.,* 1998). Future biophysical studies on the three-dimensional structure of RAP and RAP–receptor

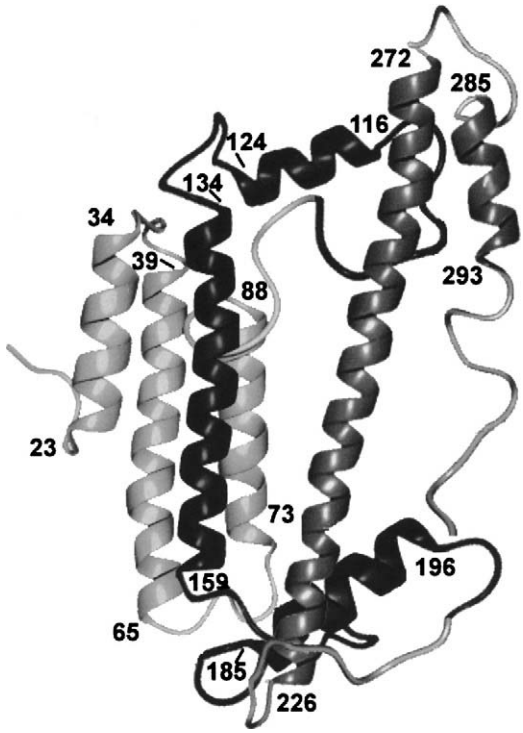



FIG. 6 One possible three-dimensional model for the structure of RAP. The model uses the NMR solution structure of repeat 1 (Nielsen *et al.,* 1997; 1NRE from the Protein Data Bank, Brookhaven National Laboratory, Upton, NY). The model also incorporates information from the limited proteolysis data and secondary structure prediction (Rall *et al.,* 1998). The potential interaction between repeats 1 and 2 to generate high-affinity LRP binding site is also illustrated (Rall *et al.,* 1998; Medved *et al.,* 1999). (From Rall, S. C. Jr., Ye, P., Bu, G., and Wardell, M. R. The domain structure of human receptor-associated protein. Protease sensitivity and guanidine HCl denaturation. *Journal of Biological Chemistry,* 1998;273:24152–24157. Reproduced with permission from The American Society for Biochemistry and Molecular Biology).

complexes will undoubtedly provide valuable information as to how this unique chaperone interacts with its target receptors and carries out its varied functions.

## VII. Concluding Remarks

While the functions of the LDLR family members have been expanded enormously within the past few years, our understanding of the biogenesis of these receptors is still limited. The chaperone functions of RAP described in this review represent a unique aspect of the LDLR family members, both in terms of their proper folding

and in consideration of their trafficking along the same secretory pathway as their ligands. Evidence has accumulated to date which clearly indicates a dual function for RAP as a molecular chaperone in promoting receptor folding and safeguarding their passage along the early secretory pathway. It is possible that RAP uses the same mechanism to maintain these receptors in an inactive state both within the cell along the secretory pathway and on the cell surface. In addition to RAP, the biogenesis of LDLR family members may well be assisted by other chaperones. These chaperones may function within the ER in concert with RAP to promote the proper folding and to assist other posttranslational events (e.g., glycosylation and degradation of misfolded proteins). In support of this hypothesis, a study by Sarti *et al.* (2000) has shown that RAP interacts with several ER resident proteins including a glycoprotein related to actin. Future biochemical, biophysical, and cellular studies will focus in more detail on how RAP carries out its unique chaperone functions and how these functions relate to those of other chaperones.

## Acknowledgments

I give my sincere thanks to Alan Schwartz for his constant support and encouragement during the past 10 years. My thanks also extend to current and former members of my laboratory and my collaborators, who have contributed to various aspects of the research described in this review. Research in the author's laboratory is supported by grants from the National Institutes of Health. The author is an Established Investigator of the American Heart Association.

## References

Abbate, M., Bachinsky, D., Zheng, G., Stamenkovic, I., McLaughlin, M., Niles, J. L., McCluskey, R. T., and Brown, D. (1993). Location of gp330/alpha 2-m receptor-associated protein (alpha 2-MRAP) and its binding sites in kidney: Distribution of endogenous alpha 2-MRAP is modified by tissue processing. *Eur. J. Cell Biol.* **61,** 139–149.

Andersen, O. M., Christensen, L. L., Christensen, P. A., Sorensen, E. S., Jacobsen, C., Moestrup, S. K., Etzerodt, M., and Thogersen, H. C. (2000). Identification of the minimal functional unit in the low density lipoprotein receptor-related protein for binding the receptor-associated protein (RAP). A conserved acidic residue in the complement-type repeats is important for recognition of RAP. *J. Biol. Chem.* **275,** 21,017–21,024.

Armstrong, P. B., Mangel, W. F., Wall, J. S., Hainfield, J. F., Van Holde, K. E., Ikai, A., and Quigley, J. P. (1991). Structure of alpha 2-macroglobulin from the arthropod Limulus polyphemus. *J. Biol. Chem.* **266,** 2526–2530.

Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S., and Strickland, D. K. (1990). The human alpha 2-macroglobulin receptor: Identification of a 420-kD cell surface glycoprotein specific for the activated conformation of alpha 2-macroglobulin. *J. Cell Biol.* **110,** 1041–1048.

Battey, F. D., Gafvels, M. E., FitzGerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, J. F., 3rd, and Strickland, D. K. (1994). The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. *J. Biol. Chem.* **269,** 23268–23273.

Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. K. (1989). The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* **341,** 162–164.

Biemesderfer, D., Dekan, G., Aronson, P. S., and Farquhar, M. G. (1993). Biosynthesis of the gp330/44-kDa Heymann nephritis antigenic complex: Assembly takes place in the ER. *Am. J. Phys.* **264,** F1011–F1020.

Birn, H., Fyfe, J. C., Jacobsen, C., Mounier, F., Verroust, P. J., Orskov, H., Willnow, T. E., Moestrup, S. K., and Christensen, E. I. (2000). Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. *J. Clin. Invest.* **105,** 1353–1361.

Birn, H., Verroust, P., Nexo, E., Hager, H., Jacobsen, C., Christiansen, E., and Moestrup, S. (1997). Characterization of an epithelial 460 kDa protein that facilitates endocytosis of intrinsic factor-vitamin B12 and binds receptor-associated protein. *J. Biol. Chem.* **272,** 26,497–26,504.

Braks, J. A., and Martens, G. J. (1994). 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. *Cell* **78,** 263–273.

Brandes, C., Novak, S., Stockinger, W., Herz, J., Schneider, W. J., and Nimpf, J. (1997). Avian and murine LR8B and human apolipoprotein E receptor 2: Differentially spliced products from corresponding genes. *Genomics* **42,** 185–191.

Brown, M. S., and Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* **232,** 34–47.

Brown, S. D., Twells, R. C., Hey, P. J., Cox, R. D., Levy, E. R., Soderman, A. R., Metzker, M. L., Caskey, C. T., Todd, J. A., and Hess, J. F. (1998). Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family. *Biochem. Biophys. Res. Commun.* **248,** 879–888.

Bu, G., Geuze, H. J., Strous, G. J., and Schwartz, A. L. (1995). 39 kDa receptor–associated protein is an ER resident protein and molecular chaperone for LDL receptor–related protein. *EMBO J.* **14,** 2269–2280.

Bu, G., Maksymovitch, E. A., Geuze, H., and Schwartz, A. L. (1994a). Subcellular localization and endocytic function of low density lipoprotein receptor-related protein in human glioblastoma cells. *J. Biol. Chem.* **269,** 29,874–29,882.

Bu, G., Maksymovitch, E. A., Nerbonne, J. M., and Schwartz, A. L. (1994b). Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. *J. Biol. Chem.* **269,** 18,521–18,528.

Bu, G., Maksymovitch, E. A., and Schwartz, A. L. (1993). Receptor-mediated endocytosis of tissue-type plasminogen activator by low density lipoprotein receptor-related protein on human hepatoma HepG2 cells. *J. Biol. Chem.* **268,** 13,002–13,009.

Bu, G., Morton, P. A., and Schwartz, A. L. (1992a). Identification and partial characterization by chemical cross-linking of a binding protein for tissue-type plasminogen activator (t-PA) on rat hepatoma cells. A plasminogen activator inhibitor type 1-independent t-PA receptor. *J. Biol. Chem.* **267,** 15,595–15,602.

Bu, G., and Rennke, S. (1996). Receptro-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. *J. Biol. Chem.* **271,** 22,218–22,224.

Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. (1992b). Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proc. Natl. Acad. Sci. USA* **89,** 7427–7431.

Bu, G. J., Rennke, S., and Geuze, H. J. (1997). ERD2 proteins mediate ER retention of the HNEL signal of LRP's receptor-associated protein (RAP). *J. Cell Sci.* **110,** 65–73.

Center, TWUGS (1998). Genome sequence of the nematode *C. elegans:* A platform for investigating biology. The C. elegans Sequencing Consortium. *Science* **282,** 2012–2018.

Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* **265,** 3116–3123.

Christensen, E. I., Birn, H., Verroust, P., and Moestrup, S. K. (1998). Membrane receptors for endocytosis in the renal proximal tubule. *Int. Rev. Cytol.* **180,** 237–284.

Czekay, R. P., Orlando, R. A., Woodward, L., Adamson, E. D., and Farquhar, M. G. (1995). The

expression of megalin (gp330) and LRP diverges during F9 cell differentiation. *J. Cell Sci.* **108,** 1433–1441.

Czekay, R. P., Orlando, R. A., Woodward, L., Lundstrom, M., and Farquhar, M. G. (1997). Endocytic trafficking of megalin/RAP complexes: Dissociation of the complexes in late endosomes. *Mol. Biol. Cell* **8,** 517–532.

Daly, N. L., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995a). Three-dimensional structure of the second cysteine-rich repeat from the human low-density lipoprotein receptor. *Biochemistry* **34,** 14474–14481.

Daly, N. L., Scanlon, M. J., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995b). Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA* **92,** 6334–6338.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M., and Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. *Neuron* **24,** 471–479.

Ellgaard, L., Holtel, T. L., Nielsen, P. R., Etzerodt, M., Gliemann, J., and Thogersen, H. C. (1997). Dissection of the domain architecture of the alpha 2-macroglobulin-receptor-associated protein. *Eur. J. Biochem.* **244,** 544–551.

Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997). Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature* **388,** 691–693.

FitzGerald, D. J., Fryling, C. M., Zdanovsky, A., Saelinger, C. B., Kounnas, M., Winkles, J. A., Strickland, D., and Leppla, S. (1995). Pseudomonas exotoxin-mediated selection yields cells with altered expression of low-density lipoprotein receptor-related protein. *J. Cell Biol.* **129,** 1533–1541.

Frykman, P. K., Brown, M. S., Yamamoto, T., Goldstein, J. L., and Herz, J. (1995). Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA* **92,** 8453–8457.

Furukawa, T., Ozawa, M., Huang, R. P., and Muramatsu, T. (1990). A heparin binding protein whose expression increases during differentiation of embryonal carcinoma cells to parietal endoderm cells: cDNA cloning and sequence analysis. *J. Biochem.* **108,** 297–302.

Gliemann, J. (1998). Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol. Chem.* **379,** 951–964.

Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., and Schneider, W. J. (1985). Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1,** 1–39.

Goretzki, L., and Mueller, B. M. (1998). Low-density-lipoprotein-receptor-related protein (LRP) interacts with a GTP-binding protein. *Bichem. J.* **336,** 381–386.

Gotthardt, M., Trommsdorff, M., Nevitt, M. F., Shelton, J., Richardson, J. A., Stockinger, W., Nimpf, J., and Herz, J. (2000). Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J. Biol. Chem.* **275,** 25,616–25,624.

Hammad, S. M., Barth, J. L., Knaak, C., and Argraves, W. S. (2000). Megalin acts in concert with cubilin to mediate endocytosis of high density lipoproteins. *J. Biol. Chem.* **275,** 12,003–12,008.

Herz, J., Clouthier, D. E., and Hammer, R. E. (1992). LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* **71,** 411–421.

Herz, J., Couthier, D. E., and Hammer, R. E. (1993). Correction: LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* **73,** 428.

Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K., and Brown, M. S. (1991). 39-kDa Protein modulates binding of ligands of low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J. Biol. Chem.* **266,** 21232–21238.

Herz, J., Gotthardt, M., and Willnow, T. E. (2000). Cellular signalling by lipoprotein receptors. *Curr. Opin. Lipidol.* **11,** 161–166.

Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. K. (1988). Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J.* **7,** 4119–4127.

Herz, J., Kowal, R. C., Goldstein, J. L., and Brown, M. S. (1990). Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* **9,** 1769–1776.

Hey, P. J., Twells, R. C., Phillips, M. S., Yusuke, N., Brown, S. D., Kawaguchi, Y., Cox, R., Guochun, X., Dugan, V., Hammond, H., Metzker, M. L., Todd, J. A., and Hess, J. F. (1998). Cloning of a novel member of the low-density lipoprotein receptor family. *Gene* **216,** 103–111.

Hiesberger, T., Hodits, R., Ullrich, R., Exner, M., Kerjaschki, D., Schneider, W. J., and Nimpf, J. (1996). Receptor-associated protein and members of the low density lipoprotein receptor family share a common epitope. An extended model for the development of passive Heymann nephritis. *J. Biol. Chem.* **271,** 28,792–28,797.

Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A., and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* **24,** 481–489.

Hobbs, H. H., Russell, D. W., Brown, M. S., and Goldstein, J. L. (1990). The LDL receptor locus in familial hypercholesterolemia: Mutational analysis of a membrane protein. *Annu. Rev. Genet.* **24,** 133–170.

Holtzman, D. M., Pitas, R. E., Kilbridge, J., Nathan, B., Mahley, R. W., Bu, G., and Schwartz, A. L. (1995). Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proc. Natl. Acad. Sci. USA* **92,** 9480–9484.

Horn, I. R., van den Berg, B. M., Moestrup, S. K., Pannekoek, H., and van Zonneveld, A. J. (1998). Plasminogen activator inhibitor 1 contains a cryptic high affinity receptor binding site that is exposed upon complex formation with tissue-type plasminogen activator. *Thromb. Haemostasis* **80,** 822–828.

Horn, I. R., van den Berg, B. M., van der Meijden, P. Z., Pannekoek, H., and van Zonneveld, A. J. (1997). Molecular analysis of ligand binding to the second cluster of complement-type repeats of the low density lipoprotein receptor-related protein. Evidence for an allosteric component in receptor-associated protein-mediated inhibition of ligand binding. *J. Biol. Chem.* **272,** 13,608–13,613.

Howell, B. W., Lanier, L. M., Frank, R., Gertler, F. B., and Cooper, J. A. (1999). The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol. Cell Biol.* **19,** 5179–5188.

Huang, W., Dolmer, K., and Gettins, P. G. (1999). NMR solution structure of complement-like repeat CR8 from the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **274,** 14,130–14,136.

Hussain, M. M., Strickland, D. K., and Bakillah, A. (1999). The mammalian low-density lipoprotein receptor family. *Annu. Rev. Nutr.* **19,** 141–172.

Iadonato, S. P., Bu, G., Maksymovitch, E. A., and Schwartz, A. L. (1993). Interaction of a 39 kDa protein with the low-density-lipoprotein-receptor-related protein (LRP) on rat hepatoma cells. *Biochem. J.* **296,** 867–875.

Innerarity, T. L., Friedlander, E. J., Rall, S. C. Jr., Weisgraber, K. H., and Mahley, R. W. (1983). The receptor-binding domain of human apolipoprotein E. Binding of apolipoprotein E fragments. *J. Biol. Chem.* **258,** 12,341–12,347.

Ishii, H., Kim, D. H., Fujita, T., Endo, Y., Saeki, S., and Yamamoto, T. T. (1998). cDNA cloning of a new low-density lipoprotein receptor-related protein and mapping of its gene (LRP3) to chromosome bands 19q12–q13.2. *Genomics* **51,** 132–135.

Jacobsen, L., Madsen, P., Moestrup, S. K., Lund, A. H., Tommerup, N., Nykjaer, A., Sottrup-Jensen, L., Gliemann, J., and Petersen, C. M. (1996). Molecular characterization of a novel human hybrid-type receptor that binds the alpha 2-macroglobulin receptor-associated protein. *J. Biol. Chem.* **271,** 31,379–31,383.

Jensen, P. H., Moestrup, S. K., and Gliemann, J. (1989). Purification of the human placental alpha 2-macroglobulin receptor. *FEBS Lett.* **255,** 275–280.

Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H. J., Suzuki, H., Kondo, H., Saeki, S., and Yamamoto, T. (1996). Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *J. Biol. Chem.* **271,** 8373–8380.

Kim, D. H., Inagaki, Y., Suzuki, T., Ioka, R. X., Yoshioka, S. Z., Magoori, K., Kang, M. J., Cho, Y., Nakano, A. Z., Liu, Q., Fujino, T., Suzuki, H., Sasano, H., and Yamamoto, T. T. (1998). A new low density lipoprotein receptor related protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes apolipoprotein E. *J. Biochem.* **124,** 1072–1076.

Kounnas, M. Z., Argraves, W. S., and Strickland, D. K. (1992). The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, alpha 2-macroglobulin receptor and glycoprotein 330. *J. Biol. Chem.* **267,** 21,162–21,166.

Kounnas, M. Z., Loukinova, E. B., Stefansson, S., Harmony, J. A., Brewer, B. H., Strickland, D. K., and Argraves, W. S. (1995). Identification of glycoprotein 330 as an endocytic receptor for apolipoprotein J/clusterin. *J. Biol. Chem.* **270,** 13,070–13,075.

Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K., and Saelinger, C. B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem.* **267,** 12,420–12,423.

Kowal, R. C., Herz, J., Goldstein, J. L., Esser, V., and Brown, M. S. (1989). Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proc. Natl. Acad. Sci. USA* **86,** 5810–5814.

Krieger, M., and Herz, J. (1994). Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63,** 601–637.

Kristensen, T., Moestrup, S. K., Gliemann, J., Bendtsen, L., Sand, O., and Sottrup-Jensen, L. (1990). Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Lett.* **276,** 151–155.

Li, Y., Marzolo, M. P., Kerkhof, P., Strous, G. J., and Bu, G. (2000). The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal For LDL receptor-related protein (LRP). *J. Biol. Chem.* **275,** 17,187–17,194.

Li, Y., van Kerkhof, P., Marzolo, M. P., Strous, G. J., and Bu, G. (2001). Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis. *Mol. Cell. Biol.* **21,** 1185–1195.

Li, Y., Wood, N., Yellowlees, D., and Donnelly, P. K. (1998). Expression of alpha 2-macroglobulin receptor-associated protein in normal human epidermal melanocytes and human melanoma cell lines. *J. Cell. Biochem.* **71,** 149–157.

Liu, C. X., Musco, S., Lisitsina, N. M., Forgacs, E., Minna, J. D., and Lisitsyn, N. A. (2000). LRP-DIT, a putative endocytic receptor gene, is frequently inactivated in non-small cell lung cancer cell lines. *Cancer Res.* **60,** 1961–1967.

Lundstrom, M., Orlando, R. A., Saedi, M. S., Woodward, L., Kurihara, H., and Farquhar, M. G. (1993). Immunocytochemical and biochemical characterization of the Heymann nephritis antigenic complex in rat L2 yolk sac cells. *Am. J. Pathol.* **143,** 1423–1435.

Mahley, R. W. (1988). Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* **240,** 622–630.

Marino, M., Zheng, G., Chiovato, L., Pinchera, A., Brown, D., Andrews, D., and McCluskey, R. T. (2000). Role of megalin (gp330) in transcytosis of thyroglobulin by thyroid cells. A novel function in the control of thyroid hormone release. *J. Biol. Chem.* **275,** 7125–7137.

Medh, J. D., Fry, G. L., Bowen, S. L., Pladet, M. W., Strickland, D. K., and Chappell, D. A. (1995). The 39-kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL receptors. *J. Biol. Chem.* **270,** 536–540.

Medved, L. V., Migliorini, M., Mikhailenko, I., Barrientos, L. G., Llinas, M., and Strickland, D. K. (1999). Domain organization of the 39-kDa receptor-associated protein. *J. Biol. Chem.* **274,** 717–727.

Misra, U. K., Gawdi, G., and Pizzo, S. V. (1999). Ligation of low-density lipoprotein receptor-related protein with antibodies elevates intracellular calcium and inositol 1,4,5-trisphosphate in macrophages. *Arch. Biochem. Biophys.* **372,** 238–247.

Moestrup, S., Kozyraki, R., Kristiansen, M., Kaysen, J. H., Rasmussen, H., Brault, D., Pontillon, F., Goda, F., Chritsensen, E., Hammond, T., and Verroust, P. (1998). The intrinsic Factor-vitamin B12 receptor and target of teratogenic antibodies is a megalin-binding peripheral membrane protein with homology to developmental proteins. *J. Biol Chem.* **273,** 5235–5242.

Moestrup, S. K., and Gliemann, J. (1991). Analysis of ligand recognition by the purified alpha 2-macroglobulin receptor (low density lipoprotein receptor-related protein). Evidence that high affinity of alpha 2-macroglobulin-proteinase complex is achieved by binding to adjacent receptors. *J. Biol. Chem.* **266,** 14,011–14,017.

Mokuno, H., Brady, S., Kotite, L., Herz, J., and Havel, R. J. (1994). Effect of the 39-kDa receptor-associated protein on the hepatic uptake and endocytosis of chylomicron remnants and low density lipoproteins in the rat. *J. Biol. Chem.* **269,** 13,238–13,243.

Muramatsu, H., Zou, K., Sakaguchi, N., Ikematsu, S., Sakuma, S., and Muramatsu, T. (2000). LDL receptor-related protein as a component of the midkine receptor. *Biochem. Biophys. Res. Commun.* **270,** 936–941.

Narita, M., Bu, G., Herz, J., and Schwartz, A. L. (1995). Two receptor systems are involved in the plasma clearance of tissue-type plasminogen activator (t-PA) *in vivo*. *J. Clin. Invest.* **96,** 1164–1168.

Narita, M., Bu, G., Olins, G. M., Higuchi, D. A., Herz, J., Broze, G. J. Jr., and Schwartz, A. L. (1995). Two receptor systems are involved in the plasma clearance of tissue factor pathway inhibitor *in vivo*. *J. Biol. Chem.* **270,** 24,800–24,804.

Neels, J. G., van Den Berg, B. M., Lookene, A., Olivecrona, G., Pannekoek, H., and van Zonneveld, A. J. (1999). The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *J. Biol. Chem.* **274,** 31,305–31,311.

Nielsen, P. R., Ellgaard, L., Etzerodt, M., Thogersen, H. C., and Poulsen, F. M. (1997). The solution structure of the N-terminal domain of $\alpha_2$-macroglobulin receptor-associated protein. *Proc. Natl. Acad. Sci. USA* **94,** 7521–7525.

Nimpf, J., and Schneider, W. J. (1998). The VLDL receptor: An LDL receptor relative with eight ligand binding repeats, LR8. *Atherosclerosis* **141,** 191–202.

Novak, S., Hiesberger, T., Schneider, W. J., and Nimpf, J. (1996). A new low density lipoprotein receptor homologue with 8 ligand binding repeats in brain of chicken and mouse. *J. Biol. Chem.* **271,** 11,732–11,736.

Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E. I., and Willnow, T. E. (1999). An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* **96,** 507–515.

Nykjaer, A., Petersen, C. M., Moller, B., Jensen, P. H., Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thogersen, H. C., Munch, M., Andreasen, P. A., and, *et al.* (1992). Purified alpha 2-macroglobulin receptor/LDL receptor-related protein binds urokinase.plasminogen activator inhibitor type-1 complex. Evidence that the alpha 2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes. *J. Biol. Chem.* **267,** 14,543–14,546.

Obermoeller, L. M., Chen, Z., Schwartz, A. L., and Bu, G. (1998). $Ca^{2+}$ and receptor-associated protein are independently required for proper folding and disulfide bond formation of the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **273,** 22,374–22,381.

Obermoeller, L. M., Warshawsky, I., Wardell, M. R., and Bu, G. (1997). Differential functions of triplicated repeats suggest two independent roles for the receptor-associated protein as a molecular chaperone. *J. Biol. Chem.* **272,** 10,761–10,768.

Oleinikov, A. V., Zhao, J., and Makker, S. P. (2000). Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin. *Biochem. J.* **347,** 613–621.

Orlando, R. A., and Farquhar, M. G. (1993). Identification of a cell line that expresses a cell surface

and a soluble form of the gp330/receptor-associated protein (RAP) Heymann nephritis antigenic complex. *Proc. Natl. Acad. Sci. USA* **90,** 4082–4086.

Orlando, R. A., and Farquhar, M. G. (1994). Functional domains of the receptor-associated protein (RAP). *Proc. Natl. Acad. Sci. USA* **91,** 3161–3165.

Orlando, R. A., Kerjaschki, D., Kurihara, H., Biemesderfer, D., and Farquhar, M. G. (1992). gp330 associates with a 44-kDa protein in the rat kidney to form the Heymann nephritis antigenic complex. *Proc. Natl. Acad. Sci. USA* **89,** 6698–6702.

Orth, K., Madison, E. L., Gething, M. J., Sambrook, J. F., and Herz, J. (1992). Complexes of tissue-type plasminogen activator and its serpin inhibitor plasminogen-activator inhibitor type 1 are internalized by means of the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *Proc. Natl. Acad. Sci. USA* **89,** 7422–7426.

Pelham, H. R. (1990). The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* **15,** 483–486.

Petersen, C. M., Ellgaard, L., Nykjaer, A., Vilhardt, F., Vorum, H., Thogersen, H. C., Nielsen, M. S., Jacobsen, C., Moestrup, S. K., and Gliemann, J. (1996). The receptor-associated protein (RAP) binds calmodulin and is phosphorylated by calmodulin-dependent kinase II. *EMBO J.* **15,** 4165–4173.

Petersen, C. M., Nielsen, M. S., Nykjaer, A., Jacobsen, L., Tommerup, N., Rasmussen, H. H., Roigaard, H., Gliemann, J., Madsen, P., and Moestrup, S.K. (1997). Molecular indentification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J. Biol. Chem.* **272,** 3599–3605.

Pietromonaco, S., Kerjaschki, D., Binder, S., Ullrich, R., and Farquhar, M. G. (1990). Molecular cloning of a cDNA encoding a major pathogenic domain of the Heymann nephritis antigen gp330. *Proc. Natl. Acad. Sci. USA* **87,** 1811–1815.

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407,** 535–538.

Pratt, W. B. (1993). The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *J. Biol. Chem.* **268.**

Rall, S. C. Jr., Ye, P., Bu, G., and Wardell, M. R. (1998). The domain structure of human receptor-associated protein. Protease sensitivity and guanidine HCl denaturation. *J. Biol. Chem.* **273,** 24152–24157.

Raychowdhury, R., Niles, J. L., McCluskey, R. T., and Smith, J. A. (1989). Autoimmune target in Heymann nephritis is a glycoprotein with homology to the LDL receptor. *Science* **244,** 1163–1165.

Rothman, J. E., and Orci, L. (1992). Molecular dissection of the secretory pathway. *Nature* **355,** 409–415.

Saito, A., Pietromonaco, S., Loo, A. K., and Farquhar, M. G. (1994). Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family. *Proc. Natl. Acad. Sci. USA* **91,** 9725–9729.

Sant, A. J., and Miller, J. (1994). MHC class II antigen processing: Biology of invariant chain. *Curr. Opin. Immunol.* **6,** 57–63.

Sarti, M., Farquhar, M. G., and Orlando, R. A. (2000). The receptor-associated protein (RAP) interacts with several resident proteins of the endoplasmic reticulum including a glycoprotein related to actin. *Exp. Cell Res.* **260,** 199–207.

Sato, A., Shimada, Y., Herz, J., Yamamoto, T., and Jingami, H. (1999). 39-KDa receptor-associated protein (RAP) facilitates secretion and ligand binding of extracellular region of very-low-density-lipoprotein receptor: Implications for a distinct pathway from low-density-lipoprotein receptor. *Biochem. J.* **341,** 377–383.

Savonen, R., Obermoeller, L. M., Trausch-Azar, J. S., Schwartz, A. L., and Bu, G. (1999). The carboxyl-terminal domain of receptor-associated protein facilitates proper folding and trafficking of the very low density lipoprotein receptor by interaction with the three amino-terminal ligand-binding repeats of the receptor. *J. Biol. Chem.* **274,** 25,877–25,882.

Scales, S. J., Gomez, M., and Kreis, T. E. (2000). Coat proteins regulating membrane traffic. *Int. Rev. Cytol.* **195,** 67–144.

Simmons, T., Newhouse, Y. M., Arnold, K. S., Innerarity, T. L., and Weisgraber, K. H. (1997). Human low density lipoprotein receptor fragment—Successful refolding of a functionally active ligand-binding domain produced in Escherichia coli. *J. Biol. Chem.* **272,** 25,531–25,536.

Simonsen, A. C., Heegaard, C. W., Rasmussen, L. K., Ellgaard, L., Kjoller, L., Christensen, A., Etzerodt, M., and Andreasen, P. A. (1994). Very low density lipoprotein receptor from mammary gland and mammary epithelial cell lines binds and mediates endocytosis of Mr 40,000 receptor associated protein. *FEBS Lett.* **354,** 279–283.

Sousa, M. M., Norden, A. G., Jacobsen, C., Willnow, T. E., Christensen, E. I., Thakker, R. V., Verroust, P. J., Moestrup, S. K., and Saraiva, M. J. (2000). Evidence for the role of megalin in renal uptake of transthyretin. *J. Biol. Chem.* **275,** 38,176–38,181.

Stefansson, S., Chappell, D. A., Argraves, K. M., Strickland, D. K., and Argraves, W. S. (1995). Glycoprotein 330/low density lipoprotein receptor-related protein-2 mediates endocytosis of low density lipoproteins via interaction with apolipoprotein B100. *J. Biol. Chem.* **270,** 19,417–19,421.

Stefansson, S., Muhammad, S., Cheng, X. F., Battey, F. D., Strickland, D. K., and Lawrence, D. A. (1998). Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **273,** 6358–6366.

Stockinger, W., Brandes, C., Fasching, D., Hermann, M., Gotthardt, M., Herz, J., Schneider, W. J., and Nimpf, J. (2000). The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. *J. Biol. Chem.* **275,** 25,625–25,632.

Stockinger, W., Hengstschlager-Ottnad, E., Novak, S., Matus, A., M, H. t., Bauer, J., Lassmann, H., Schneider, W. J., and Nimpf, J. (1998). The low density lipoprotein receptor gene family. Differential expression of two alpha 2-macroglobulin receptors in the brain. *J. Biol. Chem.* **273,** 32,213–32,221.

Strickland, D. K., Ashcom, J. D., Williams, S., Battey, F., Behre, E., McTigue, K., Battey, J. F., and Argraves, W. S. (1991). Primary structure of alpha 2-macroglobulin receptor-associated protein. Human homologue of a Heymann nephrits antigen. *J. Biol. Chem.* **266,** 13,364–13,369.

Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M., and Argraves, W. S. (1990). Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.* **265,** 17,401–17,404.

Strickland, D. K., Kounnas, M. Z., and Argraves, W. S. (1995). LDL receptor-related protein: A multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.* **9,** 890–898.

Sun, X. M., and Soutar, A. K. (1999). Expression *in vitro* of alternatively spliced variants of the messenger RNA for human apolipoprotein E receptor-2 identified in human tissues by ribonuclease protection assays. *Eur. J. Biochem.* **262,** 230–239.

Takakashi, S., Kawarabayasi, Y., Nakai, T., Sakai, J., and Yamamoto, T. (1992). Rabbit very low density lipoprotein receptor: A low density lipoprotein receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA* **89,** 9252–9256.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407,** 530–535.

Tauris, J., Ellgaard, L., Jacobsen, C., Nielsen, M. S., Madsen, P., Thogersen, H. C., Gliemann, J., Petersen, C. M., and Moestrup, S.K. (1998). The carboxy-terminal domain of the receptor- associated protein binds to the Vps10p domain of sortilin. *FEBS Lett.* **429,** 27–30.

Tomita, Y., Kim, D. H., Magoori, K., Fujino, T., and Yamamoto, T. T. (1998). A novel low-density lipoprotein receptor-related protein with type II membrane protein-like structure is abundant in heart. *J. Biochem.* **124,** 784–789.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999). Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* **97,** 689–701.

Trommsdorff, R., Borg, J. P., Margolis, B., and Herz, J. (1998). Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J. Biol. Chem.* **273,** 33,556–33,560.

Troussard, A. A., Khallou, J., Mann, C. J., Andre, P., Strickland, D. K., Bihain, B. E., and Yen, F. T. (1995). Inhibitory effect on the lipolysis-stimulated receptor of the 39-KDa receptor-associated protein. *J. Biol. Chem.* **270,** 17,068–17,071.

Umans, L., Serneels, L., Lorent, K., Dewachter, I., Tesseur, I., Moechars, D., and Van Leuven, F. (1999). Lipoprotein receptor-related protein in brain and in cultured neurons of mice deficient in receptor-associated protein and transgenic for apolipoprotein E4 or amyloid precursor protein. *Neuroscience* **94,** 315–321.

Van Uden, E., Veinbergs, I., Mallory, M., Orlando, R., and Masliah, E. (1999). A novel role for receptor-associated protein in somatostatin modulation-implications for Alzheimer's disease. *Neuroscience* **88,** 687–700.

van Vlijmen, B. J. M., Rohlmann, A., Page, S. T., Bensadoun, A., Bos, I. S. T., van Berkel, T. J. C., Havekes, L. M., and Herz, J. (1999). An extrahepatic receptor-associated protein-sensitive mechanism is involved in the metabolism of triglyceride-rich lipoproteins. *J. Biol. Chem.* **274,** 35,219–35,226.

Vash, B., Phung, N., Zein, S., and DeCamp, D. (1998). Three complement-type repeats of the low-density lipoprotein receptor-related protein define a common binding site for RAP, PAI-1, and lactoferrin. *Blood* **92,** 3277–3285.

Warshawsky, I., Bu, G., and Schwartz, A. L. (1993). 39-kD protein inhibits tissue-type plasminogen activator clearance in vivo. *J. Clin. Invest.* **92,** 937–944.

Warshawsky, I., Bu, G., and Schwartz, A. L. (1993). Identification of domains on the 39-kDa protein that inhibit the binding of ligands to the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **268,** 22,046–22,054.

Warshawsky, I., Bu, G., and Schwartz, A. L. (1994). Binding analysis of amino-terminal and carboxyl-terminal regions of the 39-kDa protein to the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **269,** 3325–3330.

Warshawsky, I., Bu, G., and Schwartz, A. L. (1995). Sites within the 39-kDa protein important for regulating ligand binding to the low-density lipoprotein receptor-related protein. *Biochemistry* **34,** 3404–3415.

Webb, J. C., Patel, DD, Jones, M., Knight, B., and Soutar, A. (1994). Characterization and tissue-specific expression of the human very low density lipoprotein (VLDL) receptor. *Hum. Mol. Genet.* **3,** 531–537.

Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407,** 527–530.

Weisgraber, K. H., Innerarity, T. L., Harder, K. J., Mahley, R. W., Milne, R. W., Marcel, Y. L., and Sparrow, J. T. (1983). The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258,** 12,348–12,354.

Weisgraber, K. H., Rall, S. C. Jr., Mahley, R. W., Milne, R. W., Marcel, Y. L., and Sparrow, J. T. (1986). Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J. Biol. Chem.* **261,** 2068–2076.

Wetterau, J. R., Aggerbeck, L. P., Rall, S. C., Jr., and Weisgraber, K. H. (1988). Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J. Biol. Chem.* **263,** 6240–6248.

Williams, S. E., Ashcom, J. D., Argraves, W. S., and Strickland, D. K. (1992). A novel mechanism for controlling the activity of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Multiple regulatory sites for 39-kDa receptor-associated protein. *J. Biol. Chem.* **267,** 9035–9040.

Willnow, T. E. (1999). The low-density lipoprotein receptor gene family: Multiple roles in lipid metabolism. *J. Mol. Med.* **77,** 306–315.

Willnow, T. E., Armstrong, S. A., Hammer, R. E., and Herz, J. (1995). Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proc. Natl. Acad. Sci. USA* **92,** 4537–4541.

Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S., and Herz, J. (1992). Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.* **267,** 26,172–26,180.

Willnow, T. E., and Herz, J. (1994). Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to Pseudomonas exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. *J. Cell Sci.* **107,** 719–726.

Willnow, T. E., Hilpert, J., Armstrong, S. A., Rohlmann, A., Hammer, R. E., Burns, D. K., and Herz, J. (1996). Defective Forebrain Development In Mice Lacking Gp330/Megalin. *Proc. Natl. Acad. Sci. USA* **93,** 8460–8464.

Willnow, T. E., Moehring, J. M., Inocencio, N. M., Moehring, T. J., and Herz, J. (1996). The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro. *Biochem. J.* **313,** 71–76.

Willnow, T. E., Rohlmann, A., Horton, J., Otani, H., Braun, J. R., Hammer, R. E., and Herz, J. (1996). RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO J.* **15,** 2632–2639.

Willnow, T. E., Sheng, Z., Ishibashi, S., and Herz, J. (1994). Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science* **264,** 1471–1474.

Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991). Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science* **252,** 1817–1822.

Wilson, D. W., Lewis, M. J., and Pelham, H. R. (1993). pH-dependent binding of KDEL to its receptor in vitro. *J. Biol. Chem.* **268,** 7465–7468.

Yamazaki, H., Bujo, H., Kusonoki, J., Seimiya, K., Kanaki, T., Morisaki, N., Schneider, W., and Saito, Y. (1996). Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. *J. Biol. Chem.* **271,** 24,761–24,768.

Yang, Z., Strickland, D. K., and Bornstein, P. (2001). Extracellular MMP2 levels are regulated by the LRP scavenger receptor and thrombospondin 2. *J. Biol. Chem.* **276,** 8403–8408.

Yochem, J., and Greenwald, I. (1993). A gene for a low density lipoprotein receptor-related protein in the nematode Caenorhabditis elegans. *Proc. natl. Acad. Sci. USA* **90,** 4572–4576.

Zhai, X. Y., Nielsen, R., Birn, H., Drumm, K., Mildenberger, S., Freudinger, R., Moestrup, S. K., Verroust, P. J., Christensen, E. I., and Gekle, M. (2000). Cubilin- and megalin-mediated uptake of albumin in cultured proximal tubule cells of opossum kidney. *Kidney Int.* **58,** 1523–1533.

Zheng, G., Bachinsky, D. R., Stamenkovic, I., Strickland, D. K., Brown, D., Andres, G., and McCluskey, R. T. (1994). Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpa 2MR, and the receptor-associated protein (RAP). *J. Histochem. Cytochem.* **42,** 531–542.

Zhuo, M., Holtzman, D. M., Li, Y., Osaka, H., DeMaro, J., Jacquin, M., and Bu, G. (2000). Role of tissue plasminogen activator receptor LRP in hippocampal long-term potentiation. *J. Neurosci.* **20,** 542–549.

# The Biology of Cortical Granules

Gary M. Wessel, Jacqueline M. Brooks, Emma Green, Sheila Haley, Ekaterina Voronina, Julian Wong, Victor Zaydfudim, and Sean Conner
Department of Molecular Biology, Cell Biology and Biochemistry,
Brown University, Providence, Rhode Island, 02912

An egg—that took weeks to months to make in the adult—can be extraordinarily transformed within minutes during its fertilization. This review will focus on the molecular biology of the specialized secretory vesicles of fertilization, the cortical granules. We will discuss their role in the fertilization process, their contents, how they are made, and the molecular mechanisms that regulate their secretion at fertilization. This population of secretory vesicles has inherent interest for our understanding of the fertilization process. In addition, they have import because they enhance our understanding of the basic processes of secretory vesicle construction and regulation, since oocytes across species utilize this vesicle type. Here, we examine diverse animals in a comparative approach to help us understand how these vesicles function throughout phylogeny and to establish conserved themes of function.

**KEY WORDS:** Cortical granule, Fertilization, Exocytosis, SNARE model, Secretion, Protein interactions, Signal transduction, Protease activity.



## I. Introduction

> The first effect of the [sperm's] approach is the sudden appearance of an envelope that is perfectly transparent and which surrounds the yellow [egg] at a distance and appears first as a circular line. I saw this envelope first appearing in the case of contact of a very small number of sperm (three or four, sometimes even one). . . . The yellow [egg] gets a kind of wrinkling on its surface and, as a result, it detaches itself at some points from the membrane which covers it; at the same time, this membrane is distended just like a bladder one would inflate and it moves away from

> the yellow [egg] which quickly becomes spherical again and its dimensions are not at all altered.
>
> Derbès, 1847 (excerpted from a translation by Kay and Shapiro, 1985)

Although Derbès did not know the origin of this envelope in his sea urchin eggs, or even its functional significance (Derbes, 1847), the description itself renews in many the excitement and dynamic process of fertilization. In the past 150 years, many have worked to explain the cellular and molecular mechanisms leading to egg activation in sea urchins and starfish, frogs, mice, and other oocytes and eggs, and because of the apparent morphogenesis of the fertilization envelope, and the ease of observing and manipulating fertilization, the sea urchin served (and remains today!) as a rich source for experimentation. Thus by the early 1900s, Lillie (1919), Just (1919), and others postulated that the fertilization envelope of sea urchins might arise from egg secretions.[1] These workers observed that formation of the fertilization envelope (FE) began at the point of sperm contact and proceeded around the circumference of the egg in a wave that was completed in about 40 s. Cortical granules, the major source of proteins and other macromolecules used to construct the fertilization envelope, were first identified and described by Harvey (1911). Evidence that the envelope derived from secretion of these vesicles appeared in studies such as by Motomura (1941), who stained sea urchin eggs with Janus green; before fertilization the cortical granules were stained, and following fertilization the envelope was stained.

We now know that the contents of cortical granules contribute to the physical and biochemical barrier that blocks polyspermy and are present in all mammals, most vertebrates, and many invertebrates (Shapiro *et al.,* 1989). Cortical granules are unique to oocytes (and mature oocytes or eggs) and are poised at, or near, the cytoplasmic face of the plasma membrane. They contain a diverse, yet discrete, population of molecules that modify the oocyte cell surface to resist additional sperm penetration (Shapiro *et al.,* 1989). These include proteases, glycosidases, cross-linking enzymes, and structural proteins. Several of the content proteins and their functions have been characterized both biochemically (Schuel, 1985; Shapiro *et al.,* 1989) and by the recent isolation of cognate cDNAs in the sea urchin (Laidlaw and Wessel, 1994; Wessel 1995; Haley and Wessel 1999), frog (Lindsay and Hedrick, pers. commun.), and mouse (Miller *et al.,* 1993).

This review will take two general perspectives. Our first perspective is the function of cortical granules in the fertilization process. The widely conserved role of cortical granules during fertilization in diverse oocytes makes it an excellent topic for comparative studies to better understand the general process of fertilization. Our second major perspective in this review is one of how cortical granules may

[1] We prefer to use the term *fertilization envelope* as used first by Derbès (1847) and others since, and not *fertilization membrane,* used also in Derbès (1847) and by others since, in order to reduce confusion with the term *plasma membrane*. The fertilization envelope is not a lipid bilayer, as implied by the term *membrane*.

serve as a model for the biology of a secretory organelle. Although oocytes in some animals are limited in the genetic manipulations possible, the unique biological features of cortical granules make it attractive for many biochemical approaches. In some cases, such as in the sea urchin, this includes their ease in isolation, *in vitro* fusion assays, morphological assay for exocytosis, and relatively large cells for manipulations.

Even though cortical granules, or similar organelles, are present in eggs of nearly all animals throughout phylogeny, the focus here is on cortical granules of a few select animals: mice, frogs, sea urchins, and starfish. Cortical granules in these animals have been intensively studied, and their eggs and oocytes show sufficient variation to assure the benefit of comparison. Other reviews on cortical granules that may be more comprehensive of specialized topics include a review specifically on mammals (Hoodbhoy and Talbot, 1994), reviews that summarize cortical granules throughout phylogeny (Guraya, 1982; Shapiro *et al.,* 1989), and a review that focuses on the ultrastructure and biogenesis of oocyte organelles (Anderson, 1974).

## II. Overview

### A. Background

Cortical granules are central to the block to polyspermy and do so by modifying the extracellular environment of the oocyte. At one extreme in mammals and in fish (Fig. 1), contents of cortical granules (cortical alveoli in fish) modify a preexisting and extensive oocyte extracellular matrix that was synthesized during oogenesis. The cortical granule contents modify the extracellular matrix in a significant and essential way, but the majority of the extracellular matrix is already intact and established prior to fertilization. At the other extreme are frogs and sea urchins. An extracellular matrix in these animals is present, but minimal. It does, however, serve as a scaffold in the construction of a new extracellular matrix by retaining and organizing the massive content secretions of the cortical granules in these animals following fertilization (Fig. 2). These two extreme roles of the cortical granules in modification of the oocyte extracellular matrix are reflected in their morphology and regulation of their contents. In this section we will summarize the generalities of cortical granule biology and then in section III we will focus in depth on four distinct oocytes and how their cortical granules function.

#### 1. Cortical Granules—the Big Picture

Cortical granules are membrane-bound organelles and members of the regulated family of secretory vesicles (Fig. 3). As their name implies, the cortical granules of an oocyte are concentrated in the cortex of the cell, the outermost few micrometers

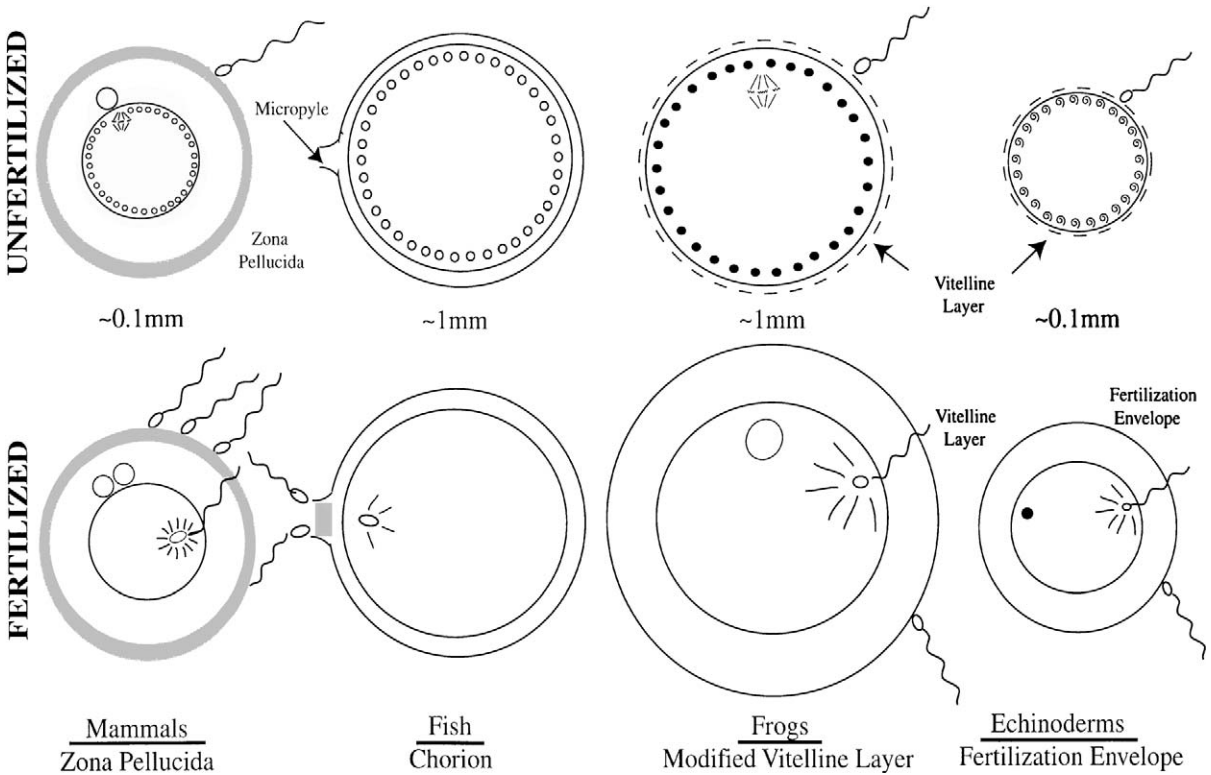



FIG. 1 Modification of the egg/oocyte extracellular matrix. Shown as representatives are mammals and fish, who have a preestablished and extensive extracellular matrix, and frogs and sea urchins, who construct the majority of their extracellular matrix from cortical granule secretions at fertilization.

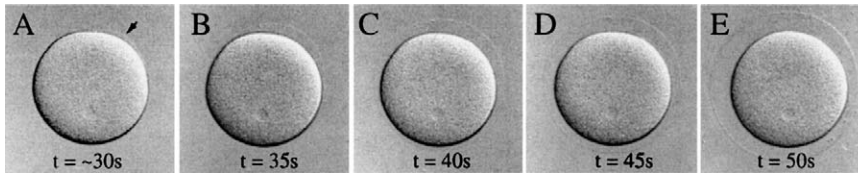



FIG. 2 Time-lapse fertilization series in the sea urchin, *Lytechinus variegates*. The eggs are approximately 100 $\mu$m in diameter and have a prominent haploid pronucleus. Arrow indicates the site of sperm fusion and the lifting/construction of the fertilization envelope. Time shown in each frame is seconds following insemination.

of the cell that is distinct both morphologically and biochemically from the inner cytoplasm. In some eggs, like those of the sea urchin, cortical granules are attached, or docked, to the plasma membrane prior to fertilization. In other cases, like in mouse oocytes, the cortical granules are concentrated near the surface of the cell, and fertilization stimulates movement to the plasma membrane and fusion.

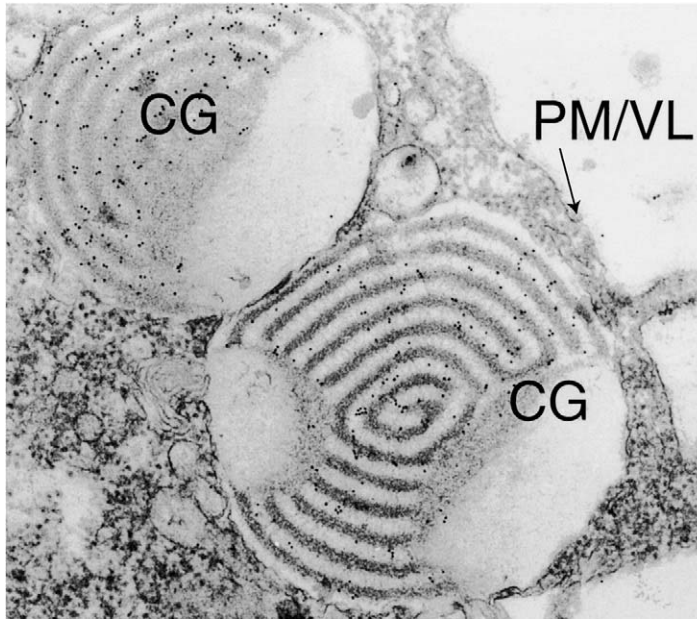



FIG. 3 Electron microscopic immunolabeling of SFE1 in eggs. Fifteen-nanometer gold particles concentrate specifically to the spiral lamellae of cortical granules when labeled with antibodies to SFE 1. Note the two fundamental regions of the cortical granule substructure: the spiral lamellae and the homogeneous region. Cortical granules are approximately 1 $\mu$m in diameter. CG, cortical granule; PM, plasma membrane; VL, vitelline layer.

Cortical granules are *similar* to regulated secretory granules in somatic cells—for example, synaptic vesicles of nerve cells and zymogen granules of pancreatic acinar cells—in that the contents are specifically packaged and concentrated into membrane-bound vesicles. These vesicles each exocytose in response to elevated cytoplasmic calcium levels initiated by an extracellular signal. The extracellular stimulus for secretion in the case of cortical granules is sperm binding, and although the exact mechanism of signal transduction is not yet known, the calcium is derived from the endoplasmic reticulum by stimulus from inositol trisphosphate (IP3). Cortical granules are *distinct,* however, from most other regulated secretory vesicles in that they are not renewed. Following fertilization cortical granules do not form again, and their contents are no longer synthesized. In fact, the mRNA encoding several of the contents of cortical granules are selectively degraded at oocyte maturation, just prior to fertilization (Laidlaw and Wessel, 1994; see below), and does not reaccumulate in development of the individual until, as a female adult, it synthesizes new oocytes.

## 2. The Function of Cortical Granules in Fertilization

One of the major functions of the contents of cortical granules is to construct or to modify the existing oocyte/egg extracellular matrix to form a biochemical and mechanical barrier that repels additional sperm (Fig. 4). Inhibiting the exocytosis of cortical granules in sea urchins definitively shows that they function in the formation of the fertilization envelope and in the block to polyspermy. Inhibiting exocytosis was accomplished using high hydrostatic pressure (Chase, 1967) and later

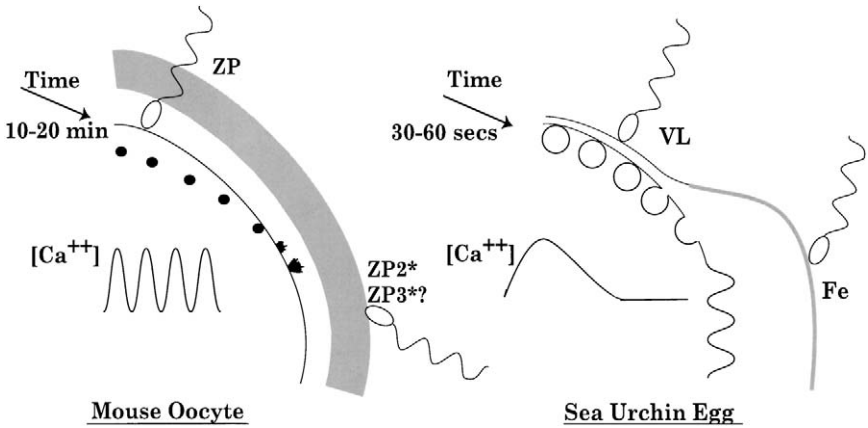



FIG. 4 Cortical granule exocytosis and modification of the egg extracellular matrix. A comparison of mouse and sea urchin egg activation is shown, including the characteristic calcium transients and flux of cortical granules in each species.

with the local anesthetics procaine, urethane, and tetracaine (Decker and Kinsey, 1983; Hylander and Summers, 1981; Longo, 1970). Anesthetic treatments of eggs dislodged the cortical granules from the cell surface so that at fertilization the cortical granules did not exocytose and an envelope did not form. In eggs thus treated, the cortical granules could readily be displaced in a centrifugal field (Hylander and Summers, 1981), not otherwise possible in untreated eggs when docked to the plasma membrane. Treated monospermic eggs still cleaved and underwent other normal events of egg activation while retaining their cortical granules within the cytoplasm. Treatment of eggs with hydrostatic pressure (between 7000 and 8000 p.s.i.) also causes detachment of the cortical granules from the plasma membrane, and following insemination, the cortical granules remained within the cytoplasm of cells devoid of a fertilization envelope (Chase, 1967). More recently, specific molecular inhibitors of the exocytosis machinery (see below; Conner and Wessel, 1998) have been used and show clearly that blocking cortical granule exocytosis leads to polyspermy.

In addition to blocking polyspermy, the modified extracellular matrix stimulated by the cortical granule contents also creates a protective barrier in which the embryo develops (Humphreys, 1967). For sea urchin and frog embryos developing in pools of open water, this extracellular layer likely acts as protection against bacteria and small eukaryotic invaders, as well as a physical buffer that dampens mechanical shock. For a mouse embryo, the cortical granule-modified zona pellucida serves to prevent implantation until the embryo traverses the oviducts and reaches the uterus. Even though the extracellular layer created or modified by the cortical granules is impervious to certain pathogens and biochemical invaders, the embryo must later be able to quickly and efficiently hatch from the extracellular matrix to feed or to implant. Specialized enzymes synthesized by the developing embryo have recently been identified that are responsible for degrading this extraembryonic matrix and allow the embryo to hatch. These enzymes have been identified in embryos such as sea urchins (Lepage *et al.,* 1992; Reynolds *et al.,* 1992; Ghiglione *et al.,* 1994; Nomura *et al.,* 1997), frogs (Katagiri *et al.,* 1997; Kitamura and Katagiri, 1998), and fish (Yasumasu *et al.,* 1992a,b), and possibly in mammals (Vu *et al.,* 1997).

## 3. Signal Transduction Leading to Cortical Granule Exocytosis

The paradigm of stimulus-dependent secretion is the synaptic vesicles of neurons, in which levels of free calcium in the presynaptic terminus reach 200 $\mu$M and the calcium is derived from extracellular fluid via voltage-gated calcium channels in the plasma membrane. Opening these channels by a change in voltage potential allows a rapid influx of exogenous calcium that causes a rapid exocytosis of docked synaptic vesicles. Following exocytosis, calcium is then rapidly pumped back out of the cell by an ATP-dependent calcium pump. Conversely, in oocytes the source of free calcium necessary for exocytosis is the endoplasmic reticulum, some of which surrounds the cortical granules (Fig. 5; Terasaki and Jaffe, 1991). The calcium

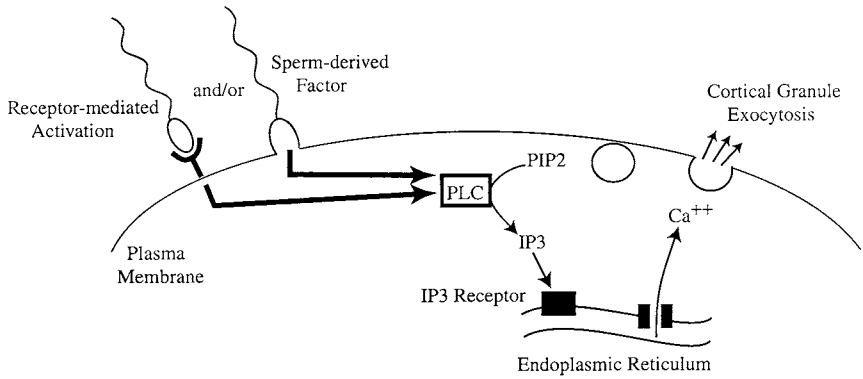



FIG. 5 Generalized model of the mechanism of intracellular calcium release leading to cortical granule exocytosis in eggs of most species studied, including mouse, frogs, and sea urchins. PLC, phospholipase C; PIP2, phosphatidylinositol bisphosphate; IP3, inositol trisphosphate.

concentration needed for exocytosis (in sea urchins) is approximately 100-fold less than needed for synaptic vesicles, on the order of 2–4 $\mu$M. The effector that stimulus calcium release from the endoplasmic reticulum is IP3, produced by phospholipase C (PLC) -mediated cleavage of phosphotidylinositol bisphosphate (PIP2). The major receptor of IP3 (IP3R), has been cloned from *Xenopus* eggs (Kume *et al.,* 1993) and shown to be an IP3-dependent calcium channel. Opening of the channel allows calcium to enter the cytoplasm by diffusion from a source of high to low concentration. The calcium concentration in the endoplasmic reticulum is enhanced by the low-affinity, high capacity calcium-binding protein calsequestrin (Lebeche and Kaminer, 1992) present in the endoplasmic reticulum. Calcium is then rapidly resequestered in the ER by the calcium-dependent ATPase pump. Thus, a wave of free cytoplasmic calcium is seen in most eggs, beginning from the point of sperm entry and propagating across the cytoplasm to the opposite pole. In some cases, as in sea urchin, a single calcium wave propagates across the cell, lasting approximately 90 s. In other eggs, however, as in mice, multiple calcium waves, or transients propagate throughout the cell over time, sometimes consisting of a dozen or more transients, lasting several hours (Stricker, 1999). How this calcium spiking is maintained is not known, but it is clear that the frequency and amplitude of the calcium transients are crucial for proper egg activation and embryonic development.

A major point of controversy is in what mechanism stimulates egg activation (Whitaker and Swann, 1993). The classic explanation is that the sperm receptor transduces a series of protein interactions and phosphorylation events resulting in PLC activation and IP3 generation. Significant circumstantial evidence supports this model, in particular, activation of eggs from the marine worm *Urechis,* with acrosomal-peptides (Gould and Stephano, 1991), and the use of exogenous

receptor activation in frog and starfish oocytes (Jaffe, 1996). In keeping with this hypothesis, one criterion for candidate receptors is the ability to transduce signals from outside the cell to the cell cytoplasm. This major qualification has recently been re-evaluated though.

An alternative mechanism for stimulating egg activation might involve direct sperm–egg cytoplasmic continuity following gamete fusion. This hypothesis has received considerable interest since the original reports that either whole sperm or sperm extracts were sufficient to induce egg activation when incorporated into the egg cytoplasm. Thus, a factor in sperm is postulated to initiate egg activation by direct cytoplasmic entry. Evidence for such a sperm factor and mechanism is now available in widespread animals, including mammals, amphibians, and echinoderms, although the identity of such factors is unknown (see Whitaker and Swann, 1993).

Recent evidence suggests that one possible sperm factor protein might be nitric oxide synthase (Kuo *et al.,* 2000). This enzyme is present in sperm cytoplasm, generates nitric oxide in the egg, and appears necessary and sufficient to activate eggs. A model emanating from this work is that nitric oxide is the mechanism for initial calcium release, which is subsequently followed by propagation signals that are mediated by IP3. Whether a nitric oxide initiating signal may be present universally in sperm is not clear, but its role in sea urchin sperm is of clear import.

## B. Perspectives

This section will introduce several different model systems used to study cortical granule biology: sea urchins, polycheates, frogs, and mice. For each organism, we will briefly describe the biology of the oocyte and the fertilization reaction. Then, in the following sections of this review, we will go into further detail of cortical granule function and regulation as we currently understand them in these diverse organisms. The goal is to understand the big picture so that we may better interpret the role, and experimentally test the function, of the cortical granules for future work.

### 1. Sea Urchins

Sea urchin eggs have been a favorite for studying the cortical reaction since Derbès' time (ca. 1847) because they are well suited for such studies. Each female sheds large numbers of haploid eggs (as many as $10^6$ per adult) approximately 100 $\mu$m in diameter. These eggs are relatively easy (and forgiving) to inject and manipulate. In addition, the cortex of the egg contains approximately 15,000 docked cortical granules and can be isolated by simple procedures, yielding cortical granule "lawns," which retain many *in vivo* properties of sperm–egg interaction and cortical granule exocytosis.

***a. Egg Jelly*** Eggs are shed externally from the ovaries, as are sperm from the male testis, resulting in external fertilization. A rapidly hydrating jelly layer surrounds the shed eggs, and acts as a sperm chemoattractant (Garbers, 1989). The major components of this jelly layer include small peptides and sulfated fucan glycoproteins, both involved in the activation of sperm. The peptides, including speract and resact, are derived from precursor proteins made by the somatic accessory cells of the ovary and are processed proteolytically to small, 10- to 14-amino-acid peptides. These peptides then diffuse out of the jelly and interact species-specifically with sperm to induce respiration and motility. The peptide receptor in sperm has been identified as a membrane-bound guanylyl cyclase; the biochemistry of sperm activation is currently being deciphered (Drewett and Garbers, 1994; Galindo *et al.,* 2000; Garbers, 1989, 1990; Nishigaki and Darszon, 2000).

The sulfated fucan glycoproteins also bind a species-specific sperm receptor, the receptor for egg jelly (REJ), causing a calcium influx that results in the acrosome reaction (Alves *et al.,* 1997, 1998; Ohbayashi *et al.,* 1998; Vacquier and Moy, 1997; Vilela-Silva *et al.,* 1999). REJ itself does not appear to be a calcium channel, but it likely interacts with and modulates a calcium channel in sperm. Biochemical examination of the structure of the glycans in egg jelly that interact with REJ suggest that some unusual transferases are involved in their biosynthesis (Kitazume *et al.,* 1996).

***b. Vitelline Layer*** Immediately apposed to the egg plasma membrane, and surrounded by the egg jelly, is the vitelline layer, a thin (approximately 10 nm) network of glycoproteins (Glabe and Vacquier, 1977; Fig. 3). The vitelline layer serves as critical scaffolding for the formation of the fertilization envelope: eggs whose vitelline layer is disrupted do not form a fertilization envelope following activation (Epel *et al.,* 1970). Approximately 25 different components are present within the vitelline layer, ranging in size from about 15 kDa to over 200 kDa (Gache *et al.,* 1983; Niman *et al.,* 1984). In contrast to the jelly layer components, each of the vitelline layer constituents appears to be synthesized and secreted by the oocyte during oogenesis, without contribution from the somatically derived accessory cells of the gonad (Chatlynne, 1969). The timing and complexity of its assembly is not entirely clear at present.

The functions and properties of the vitelline layer are complex. It provides the egg with an outer protective layer that continuously expands: gradually during growth of the oocyte and explosively during fertilization. This layer is sufficiently porous to permit transport of macromolecules into the growing oocyte and to provide binding sites for sperm attachment. Following sperm binding to the egg and exocytosis of the cortical granules, the vitelline layer provides the foundation for the attachment of cortical granule proteins such that the fertilization envelope can be assembled rapidly. Following fertilization and during early development, the vitelline layer components remain part of the fertilization envelope, which protects the embryo until it hatches at the blastula stage. Hatching of the embryo

from the fertilization envelope is catalyzed by a specific metalloprotease secreted by the presumptive ectodermal cells of the embryo (Lepage and Gache, 1989; Lepage *et al.,* 1992; Roe and Lennarz, 1990; Reynolds *et al.,* 1992).

***c. Structure of the Cortical Granules*** The major contribution to the fertilization envelope comes from the cortical granule. In echinoderms, these specialized organelles are remarkably uniform in size, structure, content, and organization, as seen by the common internal substructure of electron-dense and electron-lucent regions. In the purple sea urchin (*Strongylocentrotus purpuratus*) from the West Coast of the United States, each 1-$\mu$m-diameter cortical granule contains material arranged in a striking spiral-lamellar substructure intertwined with a nonlamellar, homogeneous region (Fig. 3). In *Lytechinus* species, on the other hand, cortical granules contain a mosaic substructure, whereas in *Arbacia* species, the granule morphology is stellate (Anderson, 1974). The different morphologies among species is even more striking considering that these vesicles contain very similar materials (Wessel, 1989). How the substructure morphology is created during granule biogenesis is not known, but it is clear that each electron-distinct region within the cortical granule contains different molecular constituents (Hylander and Summers, 1982; Alliegro and Schuel, 1988; Wessel, 1989).

***d. The Egg Cortex*** In addition to the cortical granules, the plasma membrane, and the specialized molecules involved in signal transduction during egg activation, the sea urchin egg cortex also contains an endoplasmic reticulum, large amounts of monomeric actin, and ribosomal RNA. Whether these ribosomes are in the process of translation, perhaps of proteins that function selectively in the cortex, is not yet known. The endoplasmic reticulum is present in a network that surrounds each cortical granule and is visible in thin sections under the electron microscope (Luttmer and Longo, 1985), by immunolabeling (Henson *et al.,* 1990), and in cortical preparations stained by the lipophilic dye, DiI (Terasaki *et al.,* 1991). Although the cortical endoplasmic reticulum (cER) appears contiguous with the other ER in the egg cytoplasm, it has specialized features. It contains calcium channels not seen elsewhere (Luttmer and Longo, 1985), and calsequestrin (Henson *et al.,* 1990), a protein that binds many calcium ions with low affinity. Taken together, these two proteins indicate that the cER specifically regulates the calcium necessary to trigger cortical granule exocytosis. The large amounts of monomeric actin in the egg cortex is thought to participate in both granule translocation and the elongation and stability of the microvilli following fertilization (reviewed in Longo, 1997).

Upon fertilization, the single layer of docked cortical granules fuse with the egg plasma membrane and exocytose their contents into the vitelline layer. Unlike polycheates, there are not residual granules that play a role during development; upon exocytosis, cortical granule components are permanently woven together with the vitelline components via granule-specific enzymes (see below). The resultant fertilization envelope is quite stable, and relatively unaffected by the

extracellular environment—at least until blastula-stage hatching when metalloproteases secreted by presumptive ectodermal cells begin to digest the hardened envelope so that the embryo may begin feeding (Lepage and Gache, 1989; Lepage *et al.,* 1992; Roe and Lennarz, 1990).

## 2. Polycheates

Many of the pseudocoelomates (e.g., *Caenorhabditis elegans*) or protostome invertebrates (e.g., *Drosophila melanogaster*) commonly used for genetic-based studies of embryogenesis have eggs that are fertilized internally and in these animals the mechanisms of fertilization and cortical granule function are less well understood. Recent studies of cortical granules in polycheate worms, however, have offered insight and contrast into cortical granule functions at fertilization. These include differences in structure of the cortical granules, in number and positioning of the granules in the oocyte, and even in timing secretion of the cortical granules. Polycheate sperm and eggs are shed externally, usually through ducts of the excretory organs, through short genital ducts, or even by a physiological bursting of the body wall and release of gametes. Fertilization thus occurs externally when fully grown oocytes are early in meiosis: in prophase (e.g., *Myzostoma*) or metaphase I of meiosis I (e.g., *Chaetopterus*). Cortical granules have been found in all polycheate oocytes studied to date. In particular *Arenicola, Sabellaria,* and *Nereid* species have been examined extensively (Pasteels, 1965; Bass and Brafield, 1972).

Cortical granules of polycheates differ in morphology, and number between different species. *Nereis virens* cortical granules are about 0.5 $\mu$m in diameter and are arranged in dense layers at the plasma membrane. When viewed under fluorescence microscopy swirls of unidentified fibrous material can be seen in the granules themselves (Patterson, 2001). In contrast to *N. virens* species, the elliptical cortical granules in *Arenicola marina* form a single layer at the cortex of the oocyte at prophase I.

Cortical granule exocytosis commonly occurs as a result of insemination, as is the case in Nereids, and the contents form the jelly layer outside of the vitelline layer (Fallon and Austin, 1967; Lillie, 1911). This reaction has been documented in detail by Paterson (2001), revealing that only the cortical granules at the plasma membrane take part in the mass exocytosis. In *Nereis,* layers of granules (about 15) exocytose at fertilization. This results in the production of a large extracellular jelly layer in which excess sperm become embedded. How secretion occurs is not yet known, but it is thought that content release may occur through two different pathways. The first involves ejection of membrane-bound cortical granules into the perivitelline space where they then lyse and empty their contents. In other cases, cortical granules utilize the conventional method of content exocytosis by fusing directly with the oocyte plasma membrane to which they are docked. Neither the mechanisms of content release nor the contents themselves have been identified.

In *A. marina* oocytes, which are fertilized at the prophase I stage, cortical granules line the cortex of the egg (Patterson, 2001). During this prophase I-to-metaphase I transition, germinal vesicle breakdown and cortical granule exocytosis occurs resulting in the formation of gaps in the cortex. After exocytosis, a perivitelline space forms between the oocyte plasma membrane and the vitelline layer. The vitelline layer then separates from the plasma membrane, gradually transformed into the fertilization envelope. This pattern of exocytosis and vitelline layer modification is also known to occur during spawning in another polycheate, *Sabellaria alveolata* (Pasteels, 1965).

Surprisingly, cortical granule exocytosis also occurs during maturation and spawning in *A. marina,* prior to fertilization (Patterson, 2001). This behavior is shown morphologically in well ordered oocyte stages and acts as an example to highlight the other hypothesized roles played by cortical granules. In addition to functioning in the block to polyspermy by forming an extracellular matrix, within which the now protected embryo can develop (Humphreys, 1967), certain populations of cortical granules may modify the extracellular matrix or cell surface of the oocyte prior to fertilization. In oocytes with multiple populations of cortical granules, the cell could very quickly change its surface by inserting preexisting membrane or content proteins through selective cortical granule fusion. The logistics of cortical granule regulation thus may be extra-complicated in these oocytes, making the identification of their contents and membrane proteins crucial.

## 3. Frogs

The study of fertilization and the biology of cortical granules in amphibians has been most extensive in *Xenopus,* where a substantial amount of structural and corresponding molecular information is now available. This ranges from progress in understanding the mechanisms of meiotic maturation (Barkoff *et al.,* 2000; Frank-Vaillant *et al.,* 1999; Gross *et al.,* 2000a; Mendez *et al.,* 2000), to egg activation (Runft *et al.,* 1999; Sato *et al.,* 2000), to the formation of the block to polyspermy and the recent observation of the conservation of *Xenopus* vitelline layer proteins with zona pellucida proteins of mammals (Doren *et al.,* 1999; Zhu and Naz, 1999). Indeed, the functional conservation between *Mus* zona pellucida proteins and some *Xenopus* vitelline layer proteins is sufficiently similar such that mouse zona pellucida proteins can integrate into the *Xenopus* vitelline layer—even though the two species diverged approximately 350 million years. Both conservation and the ease of isolation and utility of *Xenopus* oocytes make this cell a vital model for the future progress of fertilization studies.

*Xenopus* oocytes are shed while the cell is suspended in metaphase II of oogenesis, and sperm fusion reinitiates the oocyte meiotic program. The shed oocyte is approximately 1 mm in diameter, and surrounded by three layers of jelly (J1 through J3) that are added as the egg passes through the oviduct. The J3 layer is outermost while the J1 layer is immediately apposed to the vitelline layer. Like

the vitelline layer found in sea urchins, that in *Xenopus* is attached to, but slightly separated (~1 $\mu$m) from the plasma membrane. The *Xenopus* vitelline layer is composed of seven polypeptides, and recent progress has shown that some of these constituents are homologs of zona pellucida proteins in mammals. For example, the vitelline layer glycoprotein gp69/64 has significant sequence similarity to mammalian ZP2, and it appears to bind sperm, just as its mammalian homolog does. In addition, proteolytic modification of this ZP2 homolog appears to prevent additional sperm from binding the vitelline layer (Tian *et al.,* 1997a,b, 1999). The other mouse zona pellucida constituents, ZP1 and ZP3, appear to have functional homologs in the vitelline layer of *Xenopus* as well: gp37 is homologous to ZP1, and gp41 is homologous to ZP3 (Kubo *et al.,* 1997; Zhu and Naz, 1999).

The *Xenopus* oocyte contains two types of cortical granules, which differ from each other morphologically and geographically within the oocyte. A population of 1.5-$\mu$m granules are poised next to the plasma membrane along the entire cell surface, and in the vegetal hemisphere is an additional population of less-closely associated 2.5-$\mu$m granules. As occurs in sea urchins, fertilization initiates a wave of cortical granule exocytosis that spreads around the egg, beginning from the site of sperm contact. Secretion of both types of cortical granules relies on a mechanism similar to that found in sea urchins. Namely, exocytosis into the perivitelline space is induced by cytoplasmic calcium released from specialized cortical extensions of the endoplasmic reticulum. In *Xenopus,* this organelle is also rich in receptors for IP3 and in calsequestrin, to sequester free calcium (see above). The mechanism of signal transduction and amplification necessary to release calcium in this cell is just as unclear as in sea urchins, but likely also involves a PLC- mediated cascade (Runft *et al.,* 1999; Sato *et al.,* 2000).

The release of granule content causes the vitelline envelope to separate and lift from the plasma membrane. In contrast to the envelope formation in sea urchins, the vitelline envelope is not extensively modified in *Xenopus*. Instead, molecules from the exocytosed cortical granules diffuse through the vitelline layer and mix with the J1 layer to form a thin, electron-dense F-layer located between the J1 and the vitelline layers (Wyrick *et al.,* 1974). The major cortical granule protein responsible for the formation of the F-layer is lectin, a galactose-specific binding protein (Grey *et al.,* 1974). The interaction of jelly components in the J1 layer with this cortical granule lectin requires free calcium to precipitate from solution, as shown by double diffusion assays. The insoluble F-layer is thought to be a crucial factor that prevents other sperm from reaching the egg, but it may also act as a barrier to trap other agents (derived from cortical granules?) in the perivitelline space. With time, the vitelline layer of a fertilized egg undergoes biochemical changes, such as proteolytic digestion of gp69/64 (Gerton and Hedrick, 1986), that reduce its receptivity to sperm (Wyrick *et al.,* 1974). These biochemical changes to the vitelline layer are apparent when viewed by quick-freeze, deep etching techniques (Larabell and Chandler, 1991; Larabell and Chandler, 1988b).

As in sea urchins, the completed *Xenopus* fertilization envelope is impenetrable to sperm, and is generally resistant to exogenous proteolytic and mechanical challenges.

A protease has been identified that is possibly involved in the process of vitelline layer modification (Lindsay and Hedrick, 1995). By using protease type-specific inhibitors, it was determined that a chymotrypsin class of activity is required for vitelline layer modification, whereas a distinct, trypsin-like activity is necessary for activation of the chymotrypsin activity. When Lindsay and Hendrick isolated the chymotrypsin-like activity from activated eggs, they found a 30-kDa chymotrypsin-like species with an N-terminus amino acid sequence similar to other trypsin and chymotrypsin-like proteases. The corresponding protease cDNA revealed this protease to be part of a polyprotease translation product: it contains two trypsin-like regions and a chymotrypsin-like protease domain (Fig. 6). Furthermore, the protease domains were each separated by multiple CUB domains (see below for discussion of CUB domains). Although the protease(s) were originally modeled to be released by cortical granules during egg activation, this polyprotease has instead been localized in the perivitelline space of the inactivated egg. How the proteases are activated is yet unknown, and the functional target of the protease has yet to be determined. Preliminary evidence suggests that this ovochymase activity is not the major or direct protease responsible for gp69/64 hydrolysis, since treatment of the egg with isolated ovochymase does not lead to gp69/64 cleavage. It has been hypothesized that, perhaps, the cortical granule lectin plays some role in the protease activity—in regulating the protease or localizing its activity, for instance. Further evidence supporting this model is the recent identification of a *Xenopus* lectin homolog in the mouse (Hedrick, personal communication).

## 4. Mouse

As observed in most mammals, oocytes of the mouse are fertilized during the second metaphase of meiosis (MII). Gamete fusion then initiates the completion of meiosis. The immediate extracellular environment of an ovulated mammalian

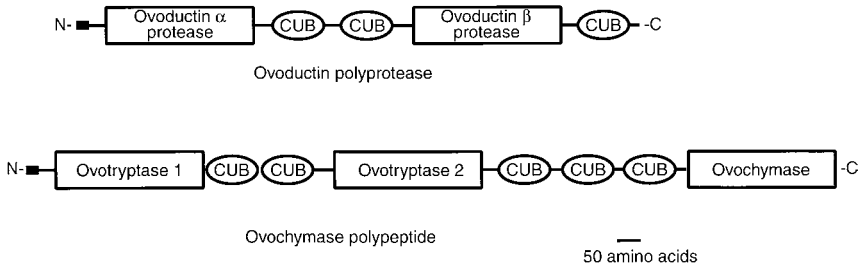



FIG. 6 Diagrammatic representation of the two polyproteases (ovoductin and ovochymase) of frog important for the fertilization reaction. CUB domains are described in the text (Section V.B.2.).

oocyte consists of an extracellular zona pellucida, which itself is surrounded by a layer of cumulus cells in a hyaluronic acid-rich matrix. Sperm must penetrate both extracellular layers to complete fertilization, but its interaction with the zona pellucida is crucial because macromolecules in this acellular layer are responsible for sperm activation.

The zona pellucida is a thick (10–15 $\mu$m) translucent layer consisting of three major proteins ZP1, ZP2, and ZP3, whose cognate genes are active only in oocytes (Epifano *et al.,* 1995; Liang *et al.,* 1997; Liang *et al.,* 1990; Rankin and Dean, 2000). This layer functions in three major events: (1) activation of the sperm acrosome reaction that elicits the sperm's ability to penetrate the oocyte; (2) the block to polyspermy (although a second major source of the block to polyspermy appears to be the plasma membrane of the egg, perhaps by fusion of cortical granules with the surface); and (3) sheltering the developing embryo until hatching enables implantation.

The general model of zona pellucida function in fertilization is that sperm bind to select carbohydrate chains on ZP3 that stimulate the acrosome reaction in sperm. The exact nature of the sperm–ZP3 interaction is controversial and may involve galactosyltransferase, a receptor tyrosine kinase, or a peripheral protein p56 (for recent reviews, see Wassarman, 1999). Once acrosome reacted, the sperm may bind to ZP2 in an intermediate step during penetration of the zona pellucida. The sperm then binds to and stimulates the cortical reaction in the egg, resulting in cortical granule exocytosis and subsequent modification of both ZP3 and ZP2. The exact biochemical modification of ZP3 is yet unclear, but functionally it means that subsequent sperm may no longer bind to nor undergo the acrosomal reaction upon contact with the fertilized egg's zona pellucida. ZP2, however, is modified sufficiently following fertilization such that it migrates more rapidly in an SDS–PAGE system (referred to as $ZP2_f$) than the original ZP2, and serves as a convenient molecular indicator of the cortical reaction (Ducibella *et al.,* 1995). The nature of this modification is not known, though it is believed to involve proteolytic cleavage by a cortical granule proteinase (Moller and Wassarman, 1989).

Cortical granules in the mouse are small (0.1–0.5 $\mu$m), electron-dense, homogeneous vesicles poised about 0.5–1.0 $\mu$m from the cell surface of the MII oocyte. They are not docked to the plasma membrane, as in sea urchins, but instead are associated with a dense, cortical microfilament array. Prior to meiotic progression, the oocyte contains a complete layer of cortical granules; after entering meiosis II, however, the cortical region associated with the meiotic spindles is devoid of cortical granules. A smooth plasma membrane is also associated with this cortical granule-free region, compared to the remaining membrane rich in microvilli. Apparently in rodent oocytes entering meiosis II, cortical granules covering the meiotic spindle exocytose prematurely, resulting in the void at the cortex and the smooth plasma membrane. Premature cortical granule exocytosis over this area also leads to premature ZP2 modification, presumably localized over the meiotic spindles: Oocytes prior to meiotic progression have no detectable $ZP2_f$, whereas

those with a cortical granule-free domain do have detectable levels of $ZP2_f$, albeit less than levels following fertilization. The alteration in membrane morphology is not completely understood, but may have a functional significance since sperm preferentially bind to and fuse at the microvillar regions of an MII oocyte (Schultz and Kopf, 1995). This premature exocytic behavior, however, is apparently a feature of only rodents since it has not been observed in any other mammalian species examined (Ducibella, 1996).

The diverse organisms listed here each exhibit differences in fertilization strategies. Yet, common to each oocyte is the presence of, and important functional requirement for, cortical granules. The remainder of the review will focus on function and regulation of cortical granule biogenesis, cortical granule contents, and regulation of exocytosis. We will use sea urchin eggs as a foundation to begin describing cortical granules, supplementing this understanding with what is known about these critical organelles in other organisms.

## III. Cortical Granule Contents and Their Function

### A. Sea Urchins

Upon fertilization in the sea urchin, cortical granules exocytose their contents, many of which modify the vitelline layer, to form a fertilization envelope (FE). The fertilization envelope lifts off the plasma membrane, generating a physical block to polyspermy (Fig. 7). Several diverse types of molecules responsible for envelope formation are synthesized and stored in the sea urchin egg. These include enzymes such as an ovoperoxidase, a protease, and a glycosidase; structural proteins such as SFE9, proteoliaisin, and SFE1; glycosaminoglycans; and perivitelline molecules such as glucanase and hyalin. Of the estimated 12 cortical granule proteins, all except two are unique to oocytes and cortical granules. The two exceptions are glucanase, which in some species is present in the digestive system during early embryogenesis and in adult life (Bachman and McClay, 1996), and hyalin, which is also present in later development, especially during gastrulation and larval development (Wessel *et al.,* 1998). Each component identified in the sea urchin cortical granule will be described in the following section.

#### 1. Ovoperoxidase

Within minutes of insemination, the fertilization envelope formed by cortical granule exocytosis hardens and becomes resistant both to mechanical and enzymatic modification. In a series of studies, Motomura (1941) suggested that this *toughening* was the result of an oxidative process. Foerder and Shapiro (1977) and Hall (1978) independently proposed that an ovoperoxidase activity is responsible for

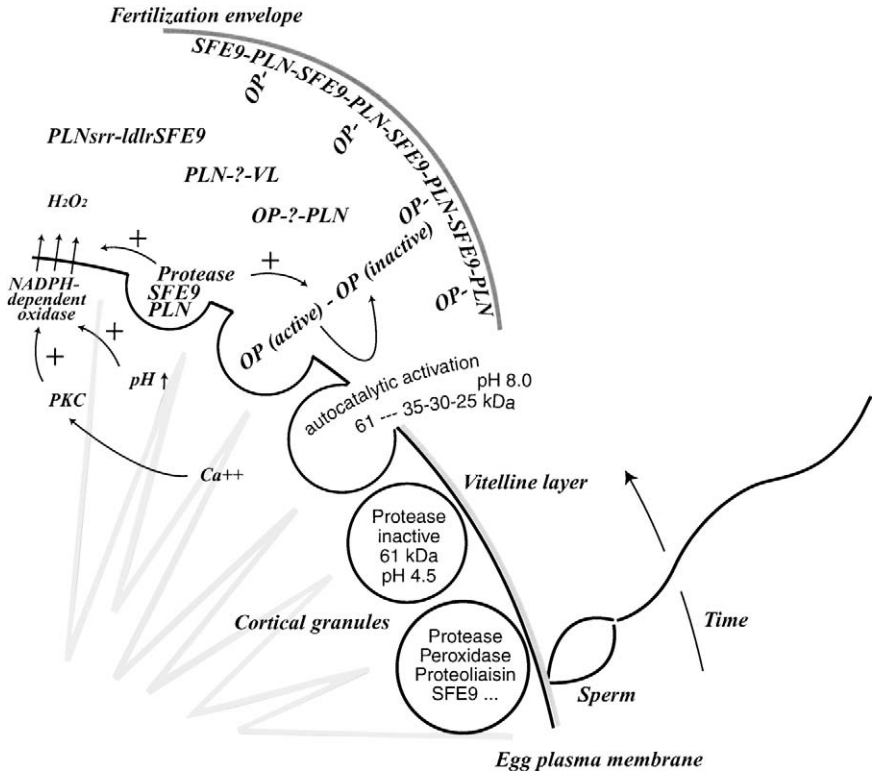



FIG. 7 Diagrammatic representation of the major elements responsible for construction of the fertilization envelope in sea urchins. See text for description of individual constituents (Section III.A). PKC, protein kinase C.

the *toughening* described by Motomura via catalysis of covalent dityrosine bonds between proteins in the fertilization envelope. Several findings support this model: (1) inhibitors of peroxidase activity inhibit hardening of the fertilization envelope (Foerder and Shapiro, 1977; Hall, 1978); (2) purified ovoperoxidase catalyzes the oxidation of tyrosine in solution (Deits *et al.,* 1984); (3) fertilization envelopes contain crosslinked di- and tri-tyrosines (Foerder and Shapiro, 1977; Hall, 1978), whereas fertilization envelopes formed following inhibition of ovoperoxidase activity have no dityrosines (Kay and Shapiro, 1987); and (4) histochemical stains for peroxidase activity and immunolabeling for the peroxidase protein show that ovoperoxidase is present in the cortical granules of unfertilized eggs and in the fertilization envelope following fertilization (Klebanoff *et al.,* 1979; Somers *et al.,* 1989).

As deduced by cDNA cloning in three species of sea urchins *Strongylocentrotus purpuratus, Lytechinus variegatus,* and *Hemicentrotus pulcherimus,* ovoperoxidase

is a heme-dependent peroxidase most similar to members of the myeloperoxidase family (LaFleur *et al.,* 1998; Nomura *et al.,* 1999). The predicted molecular weight of the *S. purpuratus* ovoperoxidase is 88.5 kDa, although biochemical purification reveals that active forms at 70 and 50 kDa are found in the FE, eggs, and exudates. The latter 50-kDa form has been shown to be a cleavage product of the 70 kDa form (Deits *et al.,* 1984), suggesting that posttranslational processing takes place, but it is not known whether these modifications alter enzyme activity or specificity. It is clear from *in situ* hybridization analysis and immunolocalizations that the ovoperoxidase is unique to oocytes and cortical granules, respectively. Within the granules of *S. purpuratus,* ovoperoxidase is localized to the spiral lamellar regions, comprises approximately 3% of the protein in the cortical granule (0.2% of total egg protein; Deits *et al.,* 1984), and is soluble upon release at fertilization (Somers *et al.,* 1989). Combined, this indicates that this peroxidase functions exclusively during fertilization. Thus, it is not similar to, or secondarily used as, a catalase system in peroxisomes of the cell, nor in the nutritive phagocytes of the ovary during destruction and removal of atretic oocytes. Instead, this peroxidase appears to have a singular function at fertilization.

Ovoperoxidase interacts with another protein of the cortical granules following exocytosis. This protein, called proteoliaisin (see below), appears to be important in targeting the ovoperoxidase to the nascent vitelline layer/fertilization envelope (Somers and Shapiro, 1991). Proteoliaisin may also protect the ovoperoxidase enzyme from proteolytic digestion (Nomura and Suzuki, 1995) and protect the cell surface from promiscuous and potentially lethal cross-linking activity. The initial interaction of ovoperoxidase with proteoliaisin is mediated by divalent cations and is reversible (Shapiro *et al.,* 1989). However, once this complex is associated with the fertilization envelope, ovoperoxidase catalyzes the covalent cross-linking of juxtaposed tyrosine residues in several high-molecular-weight proteins, including SFE 1 and SFE 9 (see below) to form a stable, macromolecular complex (Shapiro *et al.,* 1989). Each molecule of ovoperoxidase catalyzes the formation of between 5 and 10 (Kay and Shapiro, 1987) or 20 and 30 cross-links (Nomura and Suzuki, 1995), depending on the species examined. The protein targets of oxidation by ovoperoxidase appear to be specific since not all proteins are cross-linked following fertilization (Kay and Shapiro, 1987).

The oxidative substrate for ovoperoxidase catalysis is peroxide ($H_2O_2$). Peroxide is produced in the egg at fertilization by a membrane-associated NADPH-dependent oxidase that utilizes $O_2$ and $H_2O$ (Shapiro, 1991). Peroxide is generally considered a lethal oxidant, and its production at fertilization appears to be tightly regulated. The NADPH oxidase is activated *in vitro* by protein kinase C (PKC) and slightly alkaline cytoplasmic pH, both of which are rapidly induced by sperm at fertilization (Heineke and Shapiro, 1990, 1992). At least two-thirds of the oxygen consumed during the fertilization reaction, the so-called *respiratory burst* first reported by Warburg (1908), is used for peroxide production by NADPH oxidase (Shapiro, 1991).

These independent observations suggest a strong similarity to the pathway and mechanism of peroxide generation in neutrophils during phagocytosis (Klebanoff *et al.,* 1979; Shapiro, 1991). Following phagocytosis in neutrophils, small intracellular vesicles containing cytochrome $b_{558}$ (a flavohemoprotein complex of $p22^{PHOX}$ and $gp91^{PHOX}$, where PHOX stands for phagocyte oxidase) fuse with the phagosome membrane. Protein kinase C activation phosphorylates (directly?) $p47^{PHOX}$, a cytoplasmic protein in complex with $p47^{PHOX}$, $p67^{PHOX}$, and $p40^{PHOX}$ which then translocates to the cell surface, interacts with cytochrome $b_{558}$ via SH3 domains, and stimulates $O_2^-$ generation using NADPH as an electron donor [$2\ O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$] (Babior, 1999). Within the phagosome, this reactive oxygen species causes major biological damage to the engulfed pathogen. Subsequent conversion of the $O_2^-$ species to $H_2O_2$ causes additional damage, which is enhanced by the myeloperoxidase secreted into the phagosome to generate a potent antimicrobial system.

All told, the analogy to events occurring during fertilization is striking. Recall that ovoperoxidase is a myeloperoxidase-type species. The $H_2O_2$ generating system in eggs appears to depend on NADPH, which is made rapidly at fertilization due to an increase in both pH (from 6.8–7.4) and cytosolic calcium levels via a calmodulin-dependent process (Epel *et al.,* 1981). Peroxide is made within 30 s of insemination and appears to depend on protein kinase C activity, just as in neutrophils (Shapiro, 1991). Here, as in the case of glucanase (see below), a conserved pathogen defense mechanism may have been partly coopted by eggs (or vice versa) in defense not only from pathogenic invaders, but from multiple and lethal sperm fusion events. A true test of this similarity awaits identification of the molecular players in eggs.

## 2. Protease

When Hagström (1956) fertilized eggs in the presence of soybean trypsin inhibitor (SBTI), he found that the egg formed an abnormal fertilization envelope and was more susceptible to polyspermy. The envelopes that form from treated eggs did not completely detach from the plasma membrane of the SBTI-treated eggs and instead formed irregular blebs around the cell enabling additional sperm to bind and fuse with the egg. Hagström concluded that a protease was involved in a block to polyspermy in two possible ways: (1) by cleaving additional sperm receptors and (2) by cleaving connections of the vitelline layer to the plasma membrane, thereby allowing it to lift off the plasma membrane during the fertilization reaction. Vacquier *et al.* (1972) later confirmed that an activity released at fertilization by *S. purpuratus* eggs resembled that of a trypsin-like serine protease, and subsequently this activity was biochemically localized to the cortical granule (Schuel *et al.,* 1973; Vacquier *et al.,* 1973).

It is clear now that cortical granules of sea urchin eggs secrete a trypsin-like protease activity at fertilization (Alliegro and Schuel, 1988; Carroll, 1976; Decker

and Kinsey, 1983; Haley and Wessel, 1999). Carroll and Epel (1975) identified two protease activities from the cortical granule exudate, both of approximately 47 kDa. One activity proteolyses a sperm receptor (see below) referred to as the sperm receptor hydrolase, and another separates the vitelline layer from the plasma membrane, referred to as the vitelline delaminase. However, using soybean trypsin inhibitor chromatography of proteins from unfertilized eggs, other investigators identified smaller trypsin-like proteases of either 23 kDa (Fodor *et al.,* 1975), 30 kDa (Sawada *et al.,* 1984), or 35 kDa (Alliegro and Schuel, 1988). One interpretation of these disparate results is that the cortical granules contain several different protease activities resulting from different gene products. However, each investigator reported only one protease from their unfertilized egg preparations. An alternative explanation to this molecular diversity is that each of the identified proteases is encoded by one gene, but different sizes (and possible functions) result from the instability of the protein and the different methods of isolation. Using antibodies generated to the 35-kDa protease, Alliegro and Schuel (1988) localized the 35-kDa protease to cortical granules selectively by both immunofluorescence and electron microscopy. Following fertilization, the protease appears in the perivitelline space and then quickly disappears, presumably from degradation (Alliegro and Schuel, 1988). When the 35-kDa protease is isolated and used in fertilization assays, it appears to exclusively exhibit the vitelline delaminase activity: the vitelline layer of treated eggs separates from the plasma membrane (Alliegro and Schuel, 1988), while sperm were still capable of binding following vitelline layer lifting. Because disparate phyla appear to contain protease activities as an important aspect of the block to polyspermy—e.g., ovochymase in *Xenopus* (Lindsay and Hedrick, 1995) and a leupeptin-sensitive activity in mice (Moller and Wassarman, 1989)—the activity, potential substrates, and regulation of this protease may be well conserved during the fertilization reaction.

A trypsin-like protease has subsequently been cloned from cortical granules of *S. purpuratus* and key to this success was the finding that a single serine protease is present in cortical granules (Haley and Wessel, 1999). Using zymography analysis on isolated cortical granules, three major bands of activity—at 35, 30, and 25 kDa—were observed, each of which was sensitive to serine protease inhibitors, but no other inhibitor type (Haley and Wessel, 1999). These different sizes correspond to molecular weights previously reported for a single serine protease activity. Rezymography analysis determined that the lower molecular weight bands are derivatives of the larger 35-kDa band via an autocatalytic process. *S. purpuratus* cortical granule serine protease (CGSP1) has a predicted molecular weight of 61,203 Da following removal of the signal sequence, and its catalytic domain is that of a classic trypsin-like serine protease. The N-terminal half of the protein contains multiple repeats of a motif that resembles the ligand-binding domain of the LDL receptor. This structure is of particular interest since (1) it is a domain known to participate in protein interactions, (2) such motifs are present at the N-terminus of other extracellular serine proteases, and (3) this same motif is present

in several other content proteins of the cortical granules from sea urchins, often in lengthy multiples (see below).

The native substrate(s) of CGSP1 from the egg are not yet known. Since the fertilization envelope appears to retain contact with the plasma membrane when the egg is activated in the presence of SBTI, at least one likely substrate for CGSP1 is a protein that might attach to vitelline layer to the egg cell surface. Recent evidence suggests that an integrin at the surface of the egg may be cleaved by the protease (Murray *et al.,* 2000). This finding is of particular interest in light of the potential of integrins and integrin-interacting proteins in mammalian fertilization (Chen *et al.,* 1999; Miller *et al.,* 2000; Miyado *et al.,* 2000). CGSP1 may also cleave a sperm-binding protein (Foltz and Shilling, 1993; Hirohashi and Lennarz, 1998), thereby removing supernumerary sperm from the egg surface and preventing further sperm binding. Furthermore, CGSP1 may cleave other cortical granule content proteins upon exocytosis. For example, the fertilization envelope protein SFE9 appears to be proteolyzed following fertilization (Wessel, 1995). Other potential substrates of CGSP1 include precursor forms of enzymes such as ovoperoxidase, which may require cleavage before activity is achieved. Identification of the natural protease substrates in the egg will be essential to understand the extracellular modifications of the fertilization process.

## 3. Glucanase

Despite being the first exocytosed cortical granule protein identified (Epel *et al.,* 1969), the function of egg $\beta$-1,3-glucanase is still not known. The *in vitro* substrate preference of glucanase is long-chain oligosaccharides, which the enzyme cleaves at internal and external $\beta$-1,3 linkages. However, no *in vivo* substrate has yet been identified.

Most sea urchin eggs contain a $\beta$-1,3-glucanase protein, which appears to be well conserved in size (approximately 70 kDa) and epitope composition (Truschel *et al.,* 1986). In addition, embryos contain a second, but immunologically distinct form of glucanase. This second form is present selectively in the embryonic endoderm where it is presumably involved with digestion—particularly in degrading the cell walls of algae passing through the gut (Truschel *et al.,* 1986). The 70-kDa form of egg glucanase is stored in cortical granules within the lamellar region, and is secreted at fertilization (Wessel *et al.,* 1987). Unlike ovoperoxidase and structural proteins of the envelope, $\beta$-1,3-glucanase is not cross-linked within the fertilization envelope, and instead collects within the perivitelline space (see below; Wessel *et al.,* 1987). In contrast to the observation that significant glucanase activity is found in the egg following fertilization (Epel *et al.,* 1969; Muchmore *et al.,* 1969), *in situ* immunolabeling indicates that no other egg organelle contains detectable amounts of the enzyme prior to or following fertilization (Wessel *et al.,* 1987). Perhaps the egg too contains multiple glucanases which are immunologically distinct, and therefore not detectable with current reagents.

Glucanase is postulated to function in the elevation of the fertilization envelope by influencing the hydration state of the perivitelline space (Talbot and Vacquier, 1982). This hypothesis results from the reported correlations between the degree of $\beta$- 1,3-glucanase activity secreted from eggs and the extent of fertilization envelope rising that follows fertilization. However, the correlation does not hold for all echinoderms. In the sand dollar, *Dendraster excentricus,* a fertilization envelope elevates without any detectable $\beta$-1,3-glucanase activity (Vacquier, 1975a), and in species expressing the glucanase activity, inhibitors of glucanase do not affect fertilization envelope formation (Peeler *et al.,* 1987). Some evidence is available to suggest that $\beta$-1,3-glucanase could serve as a hatching enzyme or an envelope-modifying enzyme (Vacquier, 1975a), but its substrate has not yet been identified. An alternative hypothesis is that glucanase functions prior to the process of fertilization, such as in the packaging of molecules into the cortical granules (Truschel *et al.,* 1987) by virtue of its binding to, and sequestering of, select carbohydrate-linked molecules.

Bachman and McClay (1996) reported isolating the $\beta$-1,3-glucanase protein from cortical granules and, following protein microsequence, were able to identify cDNA clones and a primary sequence for the enzyme. Surprisingly, this was the first metazoan $\beta$-1,3-glucanase sequence known, and was most similar to glucanase activity from bacteria. It suggested to the authors that $\beta$-1,3-glucanase represents an ancient gene family which has subsequently undergone great divergence. Indeed, the sea urchin sequence was more similar to bacteria than to plant $\beta$-1,3-glucanase, which functions during plant defense of fungal invasion. In addition, the temperature optima and molecular size of the sea urchin glucanase is more similar to bacteria than other eukaryotes: the temperature optima for glucanase in *Bacillus circulans* and the marine eubacterium *Rhodothermus marinus* is 70 and 85°C, respectively, and 60°C for sea urchins. This is peculiar considering the animal from which the sea urchin glucanase was isolated lives under 10–15°C conditions.

Recent progress in completely unrelated processes may shed important light on the role of glucanase in cortical granules. The sequence of $\beta$-1,3-glucan-binding proteins from several invertebrates has been determined, and these too are most similar to bacterial glucan sequences. Such glucan binding proteins are present in the hemolymph of earthworms (Beschin *et al.,* 1998), silkmoths (Ochiai and Ashida, 2000), and horseshoe crabs (Seki *et al.,* 1994) where they appear to be an important part of the pathogen defense system. In the horseshoe crab hemolymph clotting process, made famous by the sensitive clinical assay for endotoxin (the limulus test, or limulus amebocyte lysate test), hemocytes degranulate in the presence of trace amounts of bacterial lipopolysaccarides (LPS; Hurley, 1995). During the diagnostic application of the limulus test, positive reactions were observed in plasma of some patients even in the absence of LPS. Since some of those patients suffered from fungal infection or were undergoing hemodialysis with cellulose dialyzers, it was concluded that a positive

reaction was caused by $\beta$-1,3-glucans. This led to reports of a $\beta$-1,3-glucan-sensitive protease zymogen in hemocyte lysate, which represents an isoform distinct from the one involved in the LPS-mediated coagulation pathway (Muta *et al.,* 1996). Recent purification of the factor causing $\beta$-1,3-glucan-stimulated hemocyte lysate gelation, referred to as factor G, revealed a protein consisting of two subunits: a $\beta$-1,3-glucan-binding protein and a serine protease zymogen. Binding of factor G to $\beta$-1,3-glucans stimulated a proteolytic cascade resulting in lysate coagulation.

This scenario may be important to our understanding of the glucanase in cortical granules and the function of cortical granule contents at fertilization. Since the cortical granules also contain a serine protease zymogen (see above), perhaps one mechanism of its activation includes a glycan-binding protein interaction. It would necessitate the presence of at least trace amounts of a $\beta$-1,3-glucan and be analogous to a conserved process of zymogen activation already present in many invertebrate defense systems. An important key to the significance of this model in sea urchin fertilization is the presence of the appropriate glycans, candidates of which have been reported repeatedly in sea urchins (Bachman and McClay, 1996; Vacquier, 1975a), but have yet to be identified.

## 4. Proteoliaisin

Proteoliaisin appears to be critical for the macromolecular self-assembly of the fertilization envelope: it tethers ovoperoxidase to the vitelline layer, and thereby restricts the potentially toxic cross-linking activity of this enzyme to the envelope (Weidman *et al.,* 1985). Due to its close proximity to ovoperoxidase, proteoliaisin is itself cross-linked into the envelope and probably contributes to its mechanical strength. Prior to fertilization, both ovoperoxidase and proteoliaisin are packaged within the spiral lamellae of the cortical granules (Somers *et al.,* 1989), suggesting that they interact with one another during the biogenesis or packaging of cortical granule contents. However, following fertilization or artificial activation of the egg missing its vitelline layer, ovoperoxidase and proteoliaisin, as well as the majority of cortical granule exudate, appears to be secreted as freely soluble and individual proteins rather than as major aggregates.

Proteoliaisin is an asymmetric molecule of about 235 kDa with a distinct globular N-terminus and a rod-shaped C-terminus (Mozingo *et al.,* 1994). Its amino acid composition is distinct: Gln/Glu, Asn/Asp, Gly, and Cys account for 50% of the residues, and of the more than 200 cysteines, *all* participate in disulfide linkages. Unlike many extracellular proteins, it has no detectable carbohydrates, potentially maximizing secondary and tertiary folding configurations of the protein. Such intramolecular interactions have not been deciphered yet, but binding studies using isolated regions of proteoliaisin suggest that is can function as a modular protein (Somers and Shapiro, 1991).

## 5. SFE 1

SFE1 is a constituent of the fertilization envelope of the sea urchin *S. purpuratus* (Laidlaw and Wessel, 1994; Wessel *et al.,* 2000) and may reveal how the envelope forms and what protein interaction domains might predominate. In the largest cDNA obtained so far, encoding approximately two-thirds of the predicted coding region of this large protein, the C-terminal half of the cognate SFE1 (soft fertilization envelope, clone 1) protein contains two different amino acid repeat motifs: a cysteine-rich (15%) motif of 40 amino acids that is tandemly repeated 22 times and is followed by a serine/threonine-rich (38%) repeat of 63 amino acids that is tandemly repeated 3.5 times. Surprisingly, just N-terminal to the cysteine-rich repeat region is a series of five repeats with similarity to the motif originally identified in the receptor of low-density lipoproteins, the LDLr motif (see below). The amino acid composition deduced from the partial SFE1 cDNA is similar also to the composition of proteoliaisin (see above), but by use of monoclonal and polyclonal antibodies to SFE1 and proteoliaisin, it is clear that they are distinct gene products (Wessel *et al.,* 2000). They may, however, have similar functions.

SFE1 is packaged selectively into the cortical granules, as are all of the cortical granule content proteins, and then is cross-linked into the fertilization envelop following fertilization. *In situ* RNA hybridization analysis shows that the mRNA of SFE1 (9 kb) is present in oocytes selectively and is turned over rapidly in the oocyte following germinal vesicle breakdown. The timing and levels of SFE1 transcript accumulation add to the general hypothesis that its cognate gene is activated concomitantly with other cortical granule-specific products. Its LDLr-like motif also may reveal a common structural mechanism responsible for protein interactions for construction of the fertilization envelope.

## 6. SFE 9

SFE 9 is another of the high-molecular-weight structural proteins cross-linked into the fertilization envelope by ovoperoxidase catalysis (Wessel, 1995). SFE 9 (soft fertilization envelope, clone 9) is packaged in the lamellar region of the cortical granules and, upon exocytosis, is released into the perivitelline space where it is quickly cross-linked into the fertilization envelope. Unlike ovoperoxidase and proteoliaisin though, SFE9 is not uniformly incorporated into the FE. Instead, it apparently binds selectively to the cast regions of the FE and not the intercast regions (Wessel, 1995). The significance of this preferential binding is not yet known.

SFE 9 and several other proteins of the fertilization envelope (see above) were identified by a cDNA screen using antibodies generated to the fertilization envelope (Laidlaw and Wessel, 1994). The predicted sequence encodes a protein of 128 kDa, containing three regions of distinct, repeating amino acid sequence. Each type of repeat is of different length, frequency, and amino acid composition, but shows

a high degree of conservation within the repeat type. A fourth region of SFE 9, present in the N-terminal region of the protein, contains an abundance of cysteines with conserved spacing. This region shows similarity to the low-density lipoprotein receptor (LDLr) motif seen in a number of proteins to mediate protein interactions (Kowal *et al.,* 1990) and in other cortical granule content proteins. SFE 9 is hypothesized to bind to the fertilization envelope via the LDLr-like region, where it is then cross-linked by ovoperoxidase to participate in the structural and biochemical block to multiple sperm penetration.

## 7. Hyalin

Hyalin is a 330-kDa fibrillar glycoprotein selectively packaged in cortical granules. It is the main constituent of the hyaline layer, an extracellular glycoprotein located internal to the fertilization envelope, but external to the apical lamina (Hall and Vacquier, 1982). Hyalin was originally isolated and characterized as a calcium-insoluble molecule released after fertilization (Yazaki, 1968; Vacquier, 1969; Kane, 1970; Citkowitz, 1971). Antibodies specific to hyalin show that the protein accumulates selectively in the cortical granules in the homogeneous region (Hylander and Summers, 1982). In addition to the egg receptor for sperm (see below), hyalin is the only other protein identified that is present in the homogeneous region.

Hyalin is distinct from other contents of the cortical granules in that it is synthesized both in the egg and during embryogenesis (Kane, 1970, 1973; Hylander and Summers, 1982; McClay and Fink, 1982). Since cortical granules are not present normally in embryos, a distinct pathway or vesicle type for hyalin secretion must be used during development. Once secreted, hyalin appears to have several important functions during development that pertain to cell interactions and morphogenesis (Citkowitz, 1971; McClay and Fink, 1982; Adelson and Humphreys, 1988). The mechanism for its activity among cells is not known, however.

Recently, cDNAs encoding hyalin have been identified, indicating that hyalin contains extensive 84-amino-acid repeats (Wessel *et al.,* 1998) and a single CUB and EGF domain at the N-terminus (W. Lennarz, pers. commun.). Using rotary shadowing, it was determined that hyalin is a filamentous molecule approximately 75 nm long with a globular "head" about 12 nm in diameter that tends to form aggregates by associating head to head in the presence of calcium (Adelson *et al.,* 1992). Hyalin molecules tended to associate with a distinct high molecular weight globular particle ("core") which maintains a tenacious association with hyalin throughout purification procedures. The epitope site(s) of the monoclonal antibody McA Tg-HYL (Adelson and Humphreys, 1988) binding to the hyalin molecule were visualized by rotary shadowing a purified hyalin–antibody complex. In these experiments, McA Tg-HYL was observed to attach to the hyalin filament near the head region in a pattern suggesting that more than one antibody binding site exists

on the hyalin filament. These multiple sites probably correspond to the hyalin repeat region, whereas the head is predicted to be the N-terminus, containing the CUB and EGF domains.

When hyalin repeats synthesized in bacteria are presented to cells in a microtiter plate adhesion assay, high-affinity binding is observed. This result suggests that carbohydrates are not solely responsible for hyalin–cell interactions, implying instead that there may be a hyalin receptor which binds one or more of the repeats. In addition, cell binding to the recombinant hyalin is inhibitable by monoclonal antibodies to the hyalin repeats. Taken together, the ultrastructural data and the cell adhesion data suggest that hyalin binds both directly to other hyalin molecules and indirectly to cell surface molecules through core particles. The orthologous repeats may act as unique cell binding sites, providing the second variable of cell receptor selectivity. Clarifying whether this specificity exists will reveal a whole new area of research comparing how cells of different stages of development utilize the same molecule.

## 8. Glycosaminoglycans

Cytochemical stains identified highly negatively charged polymers in cortical granules thought to be glycosaminoglycans (GAGs, mucopolysaccharides) (Runnstrom, 1966; Schuel *et al.,* 1974). Evidence for the presence of GAGs in cortical granules is controversial, though. Although significant amounts of hexose and hexosamine have been detected in cortical granules, which are suggestive of GAGs, uronic acid—a component of many GAGs—is undetectable (Detering *et al.,* 1977). Like classic GAGs, the cortical granule-GAG-like molecules do contain both sulfated and carboxylated groups, but appear different than most other GAGs found in the sea urchin, including those found in embryos later in development (Karp and Solursh, 1974). Thus, the exact identity of these GAG-like, negatively charged polymers is unknown.

The cortical granule GAG-like polymers are found selectively in the spiral lamellae of *S. purpuratus* and the corresponding electron-dense regions of cortical granules of other oocytes (Schuel *et al.,* 1974). Researchers speculate that the GAG-like molecules have several functions, both before and after fertilization. GAGs may participate in the biosynthesis of cortical granules, serving to condense and to concentrate the contents, and in forming aggregates of proteins destined for the cortical granules (Schuel, 1985). Within the cortical granule, the GAGs may also function to inhibit enzymatic activities. Following fertilization, rapid hydration of the GAGs may then serve to propel content exocytosis (Chandler *et al.,* 1989), and to lift the vitelline layer off the plasma membrane (Schuel, 1985). Direct evidence for any of these functions is currently lacking, but the copresence of the $\beta$-1,3-glucanase activity (see above) may be critical to deciphering the function of these unusual GAGs.

### 9. Egg Receptor for Sperm

At least some of the participants involved in the specific molecular interaction between egg and sperm have recently both been identified. *Bindin* is present on the surface of the acrosome-reacted sperm (Vacquier and Moy, 1977) and interacts with the *egg receptor for sperm,* a glycoprotein on the egg surface (Foltz and Shilling, 1993; Ohlendieck and Lennarz, 1995). Although the nature and function of the egg receptor for sperm has recently been reevaluated (see Just and Lennarz, 1997; Mauk *et al.,* 1997; Kamei *et al.,* 2000) it does appear to interact with sperm specifically through bindin. It is noteworthy that the bindin gene appears to be one of the most rapidly evolving genes known, and is believed to be one mechanism of speciation in this animal (Metz and Palumbi, 1996; Palumbi, 1999).

The *egg receptor for sperm* is concentrated on the tips of the microvilli in mature eggs as expected for its role in binding the sperm (Ohlendieck *et al.,* 1994). In addition to its sperm-recognizing extracellular domain, a comparable mass of the protein has been found within cortical granules. The cortical granule form of the receptor is in the homogeneous region of the cortical granules, colocalizing with hyalin and is secreted at fertilization. The cortical granule form of the receptor may serve as a competing, nonfunctional receptor during the fertilization reaction, acting as an "intermediate block" to polyspermy (Lillie, 1919; Ohlendieck *et al.,* 1994).

## B. Amphibians

In oocytes of the toad *Xenopus laevis*, fertilization and the cortical reaction results in the conversion of the vitelline envelope to the fertilization envelope: the vitelline layer lifts off the surface, and becomes impervious to sperm much like that seen in sea urchins. Although the details of the molecular changes are not known, operationally, the fertilization envelope becomes more resistant to solubilization by disulfide bond-reducing agents, to heat, and to proteases (Wolf, 1974a,b; Wolf *et al.,* 1976; Urch *et al.,* 1979; Bakos *et al.,* 1990). Currently, only some of the content proteins in amphibian cortical granules have been rigorously examined, and those identified appear significantly different from their counterparts in sea urchins. For example, the cortical granules do not appear to contain a peroxidase (Shapiro *et al.*, 1989) nor protease activity. Rather than requiring peroxidase-mediated disulfide cross-linking, envelope hardening appears to involve a lectin (see below) that binds to and precipitates within the J1 layer of the jelly to form the F layer. Proteolytic activity in the oocyte at fertilization also is evident with targets of this activity in the vitelline layer. The protease responsible for this activity was thought to originate from cortical granules, but it is now clear that the proteases and their activity reside instead in the perivitelline space (Lindsay and Hedrick, 1995; Lindsay *et al.,* 1999). Furthermore, this activity is a result of proteolytic

processing of a polyprotease translation product that contains the serine protease ovochymase. These proteases are inactive prior to fertilization, but gain function either by a released cortical granule content protein or by changes in a cell surface molecule induced by fertilization.

*Xenopus* cortical granules contain approximately 10 different glycoproteins, their mass ranging from 22 to 117.5 kDa (Grey *et al.,* 1976). Following fertilization, the majority of these content proteins are detectable in the perivitelline space, suggesting that they were each secreted rather than an integral part of the cortical granule membrane. At least some of these content proteins may be similar to those identified in mammals, including a lectin and an *N*-acetyl-glucosaminidase, which will be described in more detail in the following section.

### 1. Lectin

Approximately 70% of the cortical granule content protein released at fertilization in *Xenopus* is a lectin (Grey *et al.,* 1974; Greve *et al.,* 1978). It is 43-kDa, calcium-dependent, galactose-specific agglutinin (Quill and Hedrick, 1996). The lectin is present in the exudate as an aggregate, and upon its release, binds to a major 90-kDa sulfated glycoprotein found in the J1 layer (Nishihara *et al.,* 1986; Wolf *et al.,* 1976; Yurewicz *et al.,* 1975). This interaction yields a precipitate that can be detected *in vivo* as the F-layer and *in vitro* by formation of a precipitin line in double diffusion assays. Although the lectin appears to be conserved within amphibians (Hedrick and Katagiri, 1988), and specifically binds galactose containing glycoproteins, it does not appear to act species specifically. Recently, cDNA clones for the lectin have been obtained in both *Xenopus* and mouse, so we should soon have a better understanding of how this protein functions in both cortical granule biology and the fertilization reaction.

### 2. Glucosaminidase

The amphibian vitelline layer is composed of at least four glycoproteins (ZPA, ZPB, ZPC, and ZPX) that participate in sperm–egg interactions during the initial stages of fertilization. Sperm are able to bind to envelopes derived from oviposited eggs but not to those from activated eggs. The major ligand for sperm binding appears to be a complex N-linked oligosaccharide(s) containing *N*-acetylglucosamine and fucose residues conjugated to ZPC. These results are based on the observation that sperm binding is abolished after the vitelline layer components are treated with commercial $\beta$-*N*-acetylglucosaminidases, and binding was reduced by 44% after treatment with $\alpha$-fucosidase (Prody *et al.,* 1985; Vo *et al.,* 2000). Thus, one mechanism of vitelline layer modification at fertilization may be by removal of glucose and fucose on the appropriate glycoproteins.

Interestingly, cortical granules contain a $\beta$-*N*-acetylglucosaminidase activity. This enzyme runs between 37 and 40 kDa, and exhibits enzymatic characteristics

distinct form *N*-acetylglucosaminidases associated with the general endoplasmic reticulum and Golgi processing enzymes. Therefore, it appears that the cortical granule glucosaminidase activity is not simply a contaminant of the isolation process nor an outlier from poor protein trafficking. Instead, this cortical granule enzyme is responsible for an important function in the fertilization reaction.

Of special interest is the finding that terminal *N*-acetylglucosamine residues are required for sperm binding to eggs of several animals—including ascidians, mice, and pigs—and that a $\beta$-*N*-acetylglucosaminidase may remove the residue following fertilization. In ascidians, a 180-kDa, glycophosphatidylinositol-linked *N*-acetylglucosaminidase is released from the surface of eggs (ascidians lack cortical granules), where it is believed to function in the block to polyspermy by cleaving terminal *N*-acetylglucosamine residues (Downey and Lambert, 1994; Lambert *et al.,* 1997; Lambert, 1989; Lambert and Goode, 1992). Mice also release an acetylglucosaminidase from the cortical granules, which is thought to be responsible for the cleavage of terminal *N*-acetylglucosamine residues on the glycoprotein ZP3, rendering it inactive in sperm binding (see Mammals, below). By conservation alone, it is strongly believed that acetylated glucosamine plays an important role in binding and/or activating sperm and that regulation of this sugar residue could be a critical step in the process of blocking polyspermy during fertilization. This model has gained further support from *in vitro* experiments in which a significant decrease in sperm binding was observed when the vitelline layer components were treated with acetylglucosaminidase, but not with protease treatment.

## C. Mammals

The zona pellucida (ZP) in mammals is a glycoprotein matrix analogous to that of the vitelline layer in sea urchins and in amphibians. The zona pellucida is composed of three major glycoproteins, usually organized in repeating units of ZP2 and ZP3, cross-linked by ZP1. ZP3 binds sperm in a carbohydrate-mediated manner, the exact mechanism of which is uncertain (Wassarman, 1999). Sperm binding to ZP3 initiates the acrosome reaction, enabling sperm to penetrate the zona pellucida and gain access to the cell surface. Following fertilization and the cortical reaction, the zona pellucida is modified such that sperm no longer bind to nor penetrate the matrix. The only molecular change detectable in zona pellucida from fertilized eggs is the cleavage of ZP2 to a smaller form, referred to as $ZP2_f$ (Schultz and Kopf, 1995). This cleavage presumably occurs as a result of activity by a cortical granule protease, but does not appear to reflect the complete zona pellucida modification. Indeed, ZP3 is undoubtedly also modified, but in as yet unknown ways.

Although no marked morphological changes occur in the zona pellucida following fertilization, certain biochemical changes are evident. In addition to loss of sperm binding, the zona from fertilized eggs are more resistant to disulfide bond reducing agents, to low pH, to protease treatment, and to heat (Ducibella *et al.,*

1988a), much like that seen previously in the modification of the vitelline layer in frogs. This hardening reaction was postulated to use an ovoperoxidase-mediated cross-linking reaction like that seen in the sea urchin egg: peroxidase activity was reportedly associated with the fertilization reaction, and inhibitors of peroxidases appeared to abrogate the hardening phenomenon (Gulyas, 1979; Schmell and Gulyas, 1980). No compelling new evidence supports these original observations though, nor have dityrosine cross-links, reminiscent of the change seen in sea urchins, been observed. Thus, the mechanism for the observed biochemical and physical changes in the zona pellucida are still unexplained, and could perhaps be addressed if contents of the cortical granules were better understood.

Cortical granules in mice appear to contain about six major proteins, though isolation of the cortical granules is extremely difficult with limiting material in mammals. This figure is deduced from several different types of labeling experiments of the cortical granule exudate, both biosynthetic and chemical (Moller and Wassarman, 1989; Pierce *et al.,* 1990). The most recent quantitation was achieved by increasing the protein detection method by biotinylation of the proteins released from oocytes following stimulation with calcium ionomycin. The resulting detectable proteins migrated on SDS–PAGE at 70, 45, 34, 32, 28, and 20 kDa (Gross *et al.,* 2000b). Clearly some of these bands may actually represent cell surface proteins released following the treatment, and additional content proteins may be overlooked if they are too low in abundance (or low in lysine content), but as an approximation, it appears that the population of content proteins in mouse is significantly less than what is seen in sea urchins and amphibians.

Only a few of the bands detected by biotinylation have predicted functions, as listed below.

### 1. Protease

Following fertilization in mammals, ZP2 is converted from a 120 kDa protein to a 90 kDa form referred to as $ZP2_f$. This alteration is one of the major assays used to quantify fertilization, or oocyte activation and release of the cortical granules. ZP2 is believed to be hydrolyzed by a protease stored in the cortical granules (Hoodbhoy and Talbot, 1994). The protease activity was first identified by inhibition studies in hamsters, in which soybean trypsin inhibitor (SBTI) blocked conversion of the zona from a matrix of high sperm binding activity, to one with minimal binding activity. Subsequently, soybean trypsin inhibitor was used to determine the location of the protease activity in hamster oocytes. When conjugated to fluorescent markers, or to colloidal gold particles, SBTI appeared to bind to molecules within (at least some of) the cortical granules (Cherr *et al.,* 1988).

An attempt to characterize the protease was then made in mice. Following fertilization or artificial activation of oocytes, exudate (presumably enriched in cortical granule contents) was separated by HPLC and individual fractions were

assayed for their ability to cleave ZP2 within isolated zonae. An activity was found associated with a protein between 21 and 34 kDa, but was atypical of classic proteases due to its insensitivity to a variety of protease inhibitors (Moller and Wassarman, 1989). The enzymatic treatment of the zona pellucida by this fraction did, however, result in a $ZP2_f$ conversion and a zona pellucida that was resistant to acidic pH, a characteristic of zona pellucida hardening seen in many mammalian species (see above).

## 2. Glycoconjugates

The cortical granules of mouse eggs secrete certain fucosyl- and sialyl-rich glycoconjugates into the perivitelline space, which aggregate on the cell surface as embryos undergo first cleavage (Hoodbhoy *et al.,* 2000). While the cell surface of unfertilized eggs do not bind lectins, the surfaces of fertilized eggs do, which suggests that these glycoconjugates originated from cortical granules and may aid in the block to polyspermy (Hoodbhoy and Talbot, 1994).

## 3. *N*-acetylglucosamidase

Following fertilization in the mouse, ZP3 is modified by removal of certain terminal sugars, thereby releasing bound sperm and preventing further binding of additional sperm (Florman and Wassarman, 1985). The exact residue(s) hydrolyzed are under great scrutiny and disagreement, but the mechanism of cleavage likely involves the activity of a glycosidase released by the cortical granules (see discussion of the same enzyme in amphibians, above).

Mouse cortical granules contain *N*-acetylglucosamine glycosaminidase enzymes that are able to cleave *N*-acetyglucosamine from ZP3 carbohydrate chains (Miller *et al.,* 1992, 1993). This result was derived from a series of experiments including (1) detection of *N*-acetylglucosamine glycosaminidase activity (but not *N*-acetyl*galacto*samine glycosaminidase activity) in exudate of activated eggs; (2) immunoreactivity with antibodies to both $\beta$-hexosaminidase B and $\beta$-hexosaminidase A subunits in cortical granule exudate; and (3) concentrated immunoreactivity in thin sections by electron microscopy in the cortical granules. In addition, competitive inhibition of the enzyme activity during fertilization resulted in a loss of the zona block to polyspermy (Miller *et al.,* 1993). The model proposed from these results is that the *N*-acetylglucosamidase cleaves a terminal *N*-acetylglucosamine from ZP3, which would otherwise serve as a substrate for binding by a sperm cell surface galactosyltransferase. Although several other candidate proteins exist on the sperm that are postulated to be crucial for sperm binding to ZP3, this activity in the cortical granules appears to be an important candidate for its role in the block to polyspermy.

### 4. Lectin

A recent preliminary report suggested that mouse eggs may have a lectin in its cortical granules very similar to the lectin studied in amphibians (Bunnell and Hedrick, 1996). If so, then another possible dimension exists in the block to polyspermy in mammals. Recall that the lectin in amphibians is galactose-specific. Thus, if the mammalian homolog functions analogously, then exocytosis of this protein would allow it to bind to and compete with sperm for the galactose residues within the carbohydrate chains of ZP3. The galactose residues would not need be terminal, since steric hindrance can effectively block cell interactions.

### 5. 3E10

In order to screen for populations of cortical granule components, mouse oocytes were stimulated with calcium ionomycin to induce granule exocytosis, and the exudate was injected into mice to generate monoclonal antibodies. The assumption made in this experiment is that cortical granule exudate is not normally exposed to the animal, regardless of whether or not the oviduct environment is immune privileged. Several monoclonal antibodies were isolated that reacted selectively with the cortical granules by immunolocalization. One of them, termed 3E10 (the hybridoma well number), was examined more closely. It reacted against a 32-kDa protein from the cortical granule exudate, a size predicted for a content protein based on biotinylation experiments. Although no function for this 32-kDa protein has yet been determined it does appear to be specifically a cortical granule content protein (Gross *et al.,* 2000b).

## IV. Cortical Granule Protein Interactions

### A. Construction of the Fertilization Envelope in Sea Urchins

Specific protein–protein interactions are important for the molecular and biochemical events within a cell. For example, some proteins interact within an enzymatic pathway, while others serve to form structural components necessary for exerting or resisting mechanical forces. Construction of the mechanical block to polyspermy in the sea urchin is dependent upon rapid and selective protein–protein interactions among the secreted content proteins of the cortical granules and with other extracellular components. In the case of the sea urchin, were the enzymatic and structural proteins of the cortical granule unable to interact immediately with the vitelline layer scaffold, they would be rapidly diluted in the surrounding ocean rather than accomplish their intended function to block polyspermy. While only some of the interacting proteins are known from the cortical granules, several

motifs known to participate in protein–protein interactions are common to many of the fertilization envelope proteins. The following section will outline the biochemistry involved in specific protein interactions by attempting to characterize the interactions between some of the known interacting partners, and to allude to possible interactions between the molecules containing these known protein interaction motifs.

One of the first protein interactions determined among cortical granule proteins was the interaction of ovoperoxidase and proteoliaisin. Exclusively synthesized by oocytes (LaFleur *et al.,* 1998) ovoperoxidase has been previously characterized as a heme-containing, hydrogen peroxide-dependent, cross-linking enzyme that forms dityrosine bonds in the fertilization envelope (see above; Foerder and Shapiro, 1977; Hall, 1978). While this cross-linking is the major source for the overall hardening of the envelope, ovoperoxidase is believed to interact with the vitelline layer indirectly, through interactions with proteoliaisin (Somers *et al.,* 1989; Somers and Shapiro, 1991; Weidman *et al.,* 1985). Purified ovoperoxidase interacts poorly with the vitelline layer, the scaffold for cortical granule content proteins, whereas it does interact with proteoliaisin in the presence of calcium. Proteoliaisin, however, does bind to the vitelline layer also in a calcium-dependent manner (seawater contains approximately 10 mM calcium). Thus, proteoliaisin is believed to serve as a tether, or liaison, for ovoperoxidase (hence its name), localizing the enzyme's dityrosine cross-linking activity to the fertilization envelope, and potentially protecting it from proteolytic degradation (Nomura and Suzuki, 1995).

By partial proteolytic cleavage, mapping, and isolation of the proteoliaisin fragments, Somers and her coworkers identified a region of proteoliaisin that binds to ovoperoxidase, and another, distinct region that binds the vitelline layer (Somers and Shapiro, 1991). From quantitative binding studies, it appears that the vitelline layer has two distinct binding affinities for proteoliaisin. The highest affinity sites ($K_d = 0.2$ micromolar) are about six times less abundant (about $8 \times 10^7$ sites per egg) than the lower affinity sites ($K_d = 0.5$ micromolar, $5.5 \times 10^8$ sites/egg (Shapiro *et al.,* 1989)) but the molecules in the vitelline layer responsible for this binding are unknown. The reason(s) for these two affinities is not yet known, but include alternative conformations of proteoliaisin, the presence or absence of ovoperoxidase bound to proteoliaisin, or different vitelline layer components. Surprisingly, the two regions on proteoliaisin that participate in ovoperoxidase and vitelline layer interactions were found to be juxtaposed in the globular, N-terminal-most region of the protein. This leaves the distal two-thirds of proteoliaisin, or approximately 150 kDa, potentially available for dityrosine cross-linking into the envelope scaffold. This biochemical result is consistent with the interactions seen by electron microscopy of sprayed molecular complexes (Mozingo *et al.,* 1994). Even though ovoperoxidase has been cloned, it is not clear what region (or regions) interact with proteoliaisin. Unfortunately, cDNAs that encode proteoliaisin have not yet been reported, so the structures of the ovoperoxidase- and vitelline-layer-interaction domains remain to be elucidated.

This section will examine common motifs identified in the cDNA sequences of sea urchin cortical granule proteins that may help decipher how the components of cortical granules and the vitelline layer interact to form a stable envelope. We will examine the motif structures and compare them to other domains in attempt to better understand how these cortical granule proteins function.

## B. Potential Protein Interaction Motifs

### 1. LDL-Receptor-Like Motifs

An integral part of at least three separate cortical granule proteins (CGSP 1, SFE 9, and SFE 1), contains the cysteine-rich low-density lipoprotein receptor-like domain (LDLr; Fig. 8). This domain was first characterized in humans where it is responsible for the uptake of plasma lipoprotein particles and removal of cholesterol and other lipids from circulation (Brown and Goldstein, 1986; Mahley, 1988). The complete LDL receptor protein is constructed from 839 amino acids and consists of five functional LDLr domains on the N-terminal extracellular portion of the protein (Brown and Goldstein, 1986; Goldstein *et al.,* 1985). The ligand binding domain is determined by a 40-amino-acid module repeated seven times. C-terminal to this repeat is a region similar to the precursor of an epidermal growth factor (EGF), followed by a domain containing a cluster of O-linked carbohydrate chains. The C-terminal end of the molecule contains a transmembrane domain and a cytoplasmic tail important for receptor routing.

While the LDL receptor as a whole has no true application here, the ligand binding domain is of great interest. This motif is shared by many different proteins and functions in protein interactions. For example, the LDL-receptor uses this motif to bind selectively to two different apolipoproteins, apoE and apoB-100 (Goldstein *et al.,* 1985; Russell *et al.,* 1989). In other molecules, this same motif also binds to other lipid- and non-lipid-associated proteins, supporting its diverse role as an autonomous protein interaction motif.

The LDLr-like motif has been identified in a variety of molecules, some of which might be functionally similar to the proteins of the cortical granules. For example, like CGSP 1, other serine proteases contain multiple LDL-receptor like motifs, including epithin (Kim *et al.,* 1999), enterokinase (Kitamoto *et al.,* 1994), and

```
SFE 1          425 IISPCAQDEFSCGNSICIAESRHCNGYNDCYDGIDE-KNCNI--- 465
SFE 9          300 ---GCGANEFQCDTGTCIPDIQRCNNQIDCDDGSDE-ASCPIIDR 340
CGSP 1          46 QSSSCHQDESQCDDGSCIPAYLACDWYLDCSDRSDEGINCE---- 86
h LDLr         108 ---TCSQDEFRCHDGKCISRQFVCDSDRDCLDGSDE-ASCPVLTC 148
                       *  :*  *  . **.    *:   ** *  **  .*
```

FIG. 8 A comparison of single LDL-receptor cysteine-rich repeat sequences from three sea urchin cortical granule content proteins: SFE 1, SFE 9, CGSP 1, and human LDL-receptor. Cysteines, as well as acidic amino acids aspartate and glutamate are largely conserved. Alignment performed with ClustalW (Thompson *et al.,* 2000).

complement factor I (Catterall *et al.,* 1987). Perlecan, a large basement membrane-specific heparan sulfate proteoglycan core protein, identified in both human and mouse, contains four LDL-receptor like domains and participates in interactions with other basal lamina components such as collagen type IV and laminin (Murdoch *et al.,* 1992; Noonan *et al.,* 1991). Such protein interactions in the extracellular matrix are similar to what we might expect for constituents in the fertilization envelope.

To determine how this motif functions in ligand binding, two of the seven conserved cysteine-rich repeats from the human LDLr have been structurally modeled by NMR spectroscopy (Daly *et al.,* 1995a,b). Each of the seven 40-amino-acid repeats was shown to contain six cysteines, which form three disulfide bonds, as well as a cluster of negatively charged amino acids—aspartate and glutamate, adjacent to a serine (Fig. 9). Subsequently, the crystal structure suggested disulfide bonding between cysteine 1 and 3, 2 and 5, and 4 and 6; the sixth cysteine is flanked by the negatively charged residues, thought to be involved in ligand binding, and disulfide bonding between domains of the cysteine-rich repeats has been excluded by peptic cleavage experiments (Bieri *et al.,* 1995, 1998). Recent studies have suggested a minimal binding domain of LDLr. In the multifunctional chicken oocyte receptor for yolk deposition (a LDLr homolog), a polypeptide minireceptor consisting of 80 amino acids and containing 12 cysteines, two regions of negative charge density (EDGSDE and DSGEDEE) were shown to be sufficient for ligand interaction (Bajari *et al.,* 1998). This result implies that the multiple LDLr motifs seen in proteins may bind different ligands, with different affinities, offering a subtle complexity of protein interaction possibilities.

The LDL-receptor-like domains found in cortical granule proteins of the sea urchin are present in different copy numbers. While the human LDL receptor has 7 domains, SFE 1 has 22, SFE 9 has 14, and CGSP 1 has 5. In each case, the 40 amino acid repeats show similarity to each other as well as to the human LDL receptor (Fig 8). While the exact amino acid sequence of the compared motifs is different, the cysteines that impart the loop-like conformation to the motif via disulfide bonding and the acidic amino acids responsible for the ligand binding domain are highly conserved. Even in the human LDL receptor, each of the cysteine-rich repeats show significant sequence heterogeneity, yet the positioning of the cysteine and acidic residues and the predicted loop-like structures appear to be highly conserved. Furthermore, the LDL-receptor-like domains in each of these cortical granule proteins are located at the N-terminus of the protein, as in the original LDL receptor.

As shown by X-ray crystallography, the correct folding of the human LDL-receptor repeats *in vitro* is dependent upon the presence of calcium (Bieri *et al.,* 1998; Fass *et al.,* 1997). This property is consistent with the postfertilization environment of the sea urchin fertilization envelope, and the demonstrated calcium sensitivity of envelope formation and specific protein interaction, e.g., proteoliaisin–vitelline layer and proteoliaisin–ovoperoxidase. The residues important for calcium

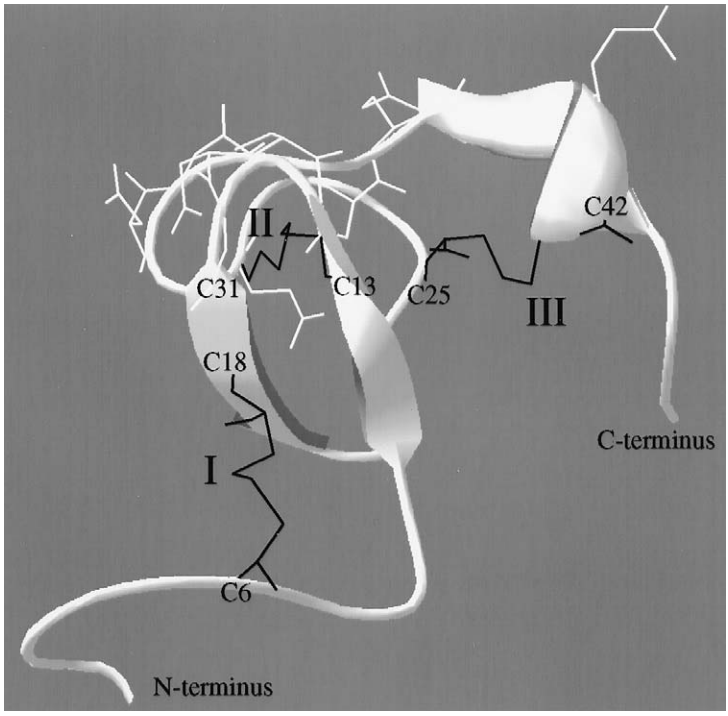



FIG. 9 The structure of human LDL-receptor repeat number 2 module involved in ligand binding. Original structure was obtained from SWISS-MODEL (http://www.expasy.ch/swissmod/SWISS-MODEL.html developed by Manuel C. Peitsch and Nicolas Guex) and labeled 11LDR.pdb in their database was then first modeled with Swiss-Pdb Viewer (http://www.expasy.ch/spdbv/mainpage.html developed by Nicolas Guex, Alexandre Diemand, Torsten Schwede, and Manuel C. Peitsch). Later renditions were made with Adobe Illustrator 8.0.

binding in the human LDL receptor were shown to be the same acidic residues previously thought to be important in ligand interactions, suggesting that the highly conserved cysteine composition and acidic sequences are important for the proper folding of the LDL-receptor motif and for ligand binding within the envelope.

Although the putative protein partners for the LDLr motif of cortical granule proteins are currently unknown, we may be able to extrapolate predicted structural motifs based on what is known about LDLr function in other animals. The ligands for the LDLr in humans are well-characterized: (Mahley, 1988; Russell *et al.,* 1989) apolipoprotein E (apoE, $M_r$ 34,000 Da) (Weisgraber, 1994) and apolipoprotein B-100 (apoB100, $M_r \sim 550{,}000$ Da) (Schumaker *et al.,* 1994). Both apolipoproteins are responsible for the transport and uptake of lipids and cholesterol via high-affinity interaction with various cellular receptors including the LDL receptor. Early studies indicated that the LDLr has a 10-fold greater affinity for apoE, even though apoB100 is more than 10 times larger (Innerarity and Mahley, 1978). The

region of apoE responsible for binding to LDLr has also been determined, and been pinpointed in this 299-amino-acid protein to be between residues 126 and 191 (Innerarity *et al.,* 1983; Weisgraber *et al.,* 1983). The region of contact is directly centered around residues 136–150. This region is enriched in basic residues (Arg–Lys–His), and is postulated to interact with the outwardly facing acidic residues on the receptor (Mahley, 1988). The subsequent crystal structure of the LDL-receptor binding motif of apoE showed an elongated four-helix bundle with a hydrophobic core and clustering of LDL-receptor-binding, basic residues on the surface of one of the helices (Wilson *et al.,* 1991).

To date, none of the cortical granule proteins show sequence similarity to the apo E or apo E motif, so we can only speculate about the function of the LDLr motifs in the sea urchin cortical granule proteins. SFE 1 and SFE 9 appear to be structural proteins lacking enzymatic activity, so it may be that the LDL-receptor domain contributes to intermolecular interactions between the secreted cortical granule proteins and/or vitelline layer during fertilization envelope formation (Wessel, 1995). In CGSP 1, the motif may serve to mediate substrate specificity, or to stabilize enzymatic activity (Haley and Wessel, 1999). In addition, disulfide bonded loops have been shown to play a role in protein sorting in the *trans*-Golgi network (Cool *et al.,* 1995), so the LDL-receptor-like repeats may also play a role in selectively sorting the proteins before they are packaged into cortical granule. Keeping an eye to structural motifs, not just sequences, that are similar to LDLr ligands will be critical—especially when we consider that the sequence of LDLr repeats even within a protein can vary widely.

## 2. CUB Motif

Sea urchin embryos were one of the original organisms in which CUB domains were characterized (Bork and Beckmann, 1993), so it is not surprising to see the ubiquity of this motif in the sea urchin egg. The name CUB is an acronym originating from the names of the first three identified proteins of the family: complement components CIR/CIS (Journet and Tosi, 1986), sea urchin fibropellins Uegf (Bisgrove and Raff, 1993), and bone morphogenetic protein 1 BMP 1 (Bork and Beckmann, 1993). CUB domains are regions of proteins ~110 amino acids in length, the structure of which suggests an antiparallel beta-barrel similar to those in immunoglobulins. As with the LDLr motif, the predicted structure of the CUB motif is conserved, whereas, with the exception of four conserved cysteines that are likely involved in the formation of disulfide bridges, sequence divergence between CUB domains is great. Although the majority of CUB domain-containing proteins are involved in development, the specific role of this domain is still poorly defined.

The first suggested role of this primarily extracellular domain comes from studies on spermadhesin (Romao *et al.,* 1997; Varela *et al.,* 1997). The small size of spermadhesin proteins (109–133 residues) practically renders them a functional CUB domain. Since this CUB protein serves as a receptor, it was expected that its ligand(s) might provide us with clues about CUB-domain-binding partners and,

therefore, the function of this domain. The most promiscuous, but least informative, partner was found to be another CUB domain (Romao *et al.,* 1997; Varela *et al.,* 1997). Other candidate ligands of spermadhesin, however, suggested some functions: CUB domains may have a role in mediating sperm–egg interactions, possibly allowing binding of spermadhesins to various glycoconjugated motifs on the egg envelope (Dostalova *et al.,* 1995a; Topfer-Petersen *et al.,* 1998). CUB domains have also been found to bind heparin (Sanz *et al.,* 1993), serine protease inhibitors (Sanz *et al.,* 1992), or phosphorylethanolamine (Dostalova *et al.,* 1995b), suggesting that these domains play a protective function toward the cell from which they were secreted.

To date, there are five sea urchin proteins known to contain a CUB domain. Fibropellin I and fibropellin III, components of the extracellular matrix, both contain a CUB domain flanked by EGF domains. However, while fibropellin III is at low levels during fertilization and early cleavage, reaching abundance after late morula (Bisgrove and Raff, 1993), fibropellin I is secreted following fertilization and contributes to the apical lamina (Bisgrove *et al.,* 1991). The other proteins are novel and have yet to be fully characterized.

It is interesting that fibropellin I has been localized to the apical lamina since a CUB domain has recently been identified in the sea urchin cortical granule content protein hyalin (M. Just and W. Lennarz, pers. commun.), supporting the CUB–CUB interaction hypothesis first observed in spermadhesin (Romao *et al.,* 1997; Varela *et al.,* 1997). Hyalin is a fibrillar protein of ~330 kDa, which is known for its high degree of adherence to itself as well as other components of the perivitelline layer (see above; Wessel *et al.,* 1998). The cDNA of hyalin encodes 37 tandemly arranged mucin repeats of 84 residues that seems to suggest a binding site for cell adhesion. While cell–hyalin interactions have been previously proposed to be involved in morphogenic movements (Adelson and Humphreys, 1988; Fink and McClay, 1985; McClay and Fink, 1982), the mechanism of this interaction has not been determined. The recent discovery of the CUB domains in hyalin may help solve this conundrum. Hyalin, like many other CUB-containing proteins, has a single CUB domain adjacent to an EGF domain; in hyalin, it is sandwiched between the EGF repeats and the 37 tandem mucin repeats (M. Just and W. Lennarz, pers. commun.). The significance of its conserved EGF positioning has not been investigated yet, although this observation may be a key that unlocks the role of hyalin, and CUB domains in general, during the block to polyspermy.

## V. Gene Regulation and RNA Turnover in Oocytes: Oocyte-Specific Gene Transcription

One of the central issues in developmental biology concerns the molecular mechanisms whereby a multipotent cell gives rise to distinct progeny. This differentiation is evidenced by the specific expression of subsets of proteins in some cells and

their absence in others, which reflects variations in their pattern of gene expression. Recently, an increasing number of genes that are expressed exclusively during oogenesis, and that support fertilization and early zygotic development, have been described.

Biogenesis of the sea urchin vitelline layer–fertilization envelope and the mouse ZP serve the same purpose: they create a unique protective environment necessary to support the oocyte during its maturation, prepare it for successful fertilization, and ensure the developing zygote a stable environment to support its growth. Transcription of the components that make up these layers appears to be coordinately regulated, and is restricted to the growth phase of oogenesis. The following section will examine the timing and regulated expression of the genes necessary for these layers to exist.

## A. Sea Urchin Vitelline Layer

The sea urchin fertilization envelope is woven from both the egg vitelline layer and the contents of the cortical granules. The vitelline layer components are believed to be synthesized and directly secreted by the oocyte during oogenesis. At fertilization, the cortical reaction causes a dramatic morphological remodeling of this layer by expulsion of content proteins that modify the vitelline layer by enzymatically separating it from the egg plasma membrane and by adding structural proteins that help the envelope lift away. Other than hyalin and glycosaminoglycans, which are found both in egg cortical granules and in developing embryos (see above), *de novo* synthesis of cortical granule proteins is restricted to oocytes. Coordinate with this protein expression pattern is an accumulation in mRNA to high levels (cortical granule content proteins can, in several cases, accumulate to 2% of the total mRNA population of the oocyte), where transcripts are first detected during early oogenesis, accumulate during oocyte maturation, quickly decline at oocyte maturation, and cannot be detected in fertilized eggs or embryos (Laidlaw and Wessel, 1994).

This transcriptional burst is confined to the growth phase of oogenesis since rapid degradation of mRNA-encoding content proteins of the cortical granules occurs upon oocyte maturation (Laidlaw and Wessel, 1994). Late germinal vesicle stage oocytes have maximal levels of each mRNA per cell, whereas mature eggs show only background levels, as determined by both *in situ* mRNA hybridization and RNA gel blots. This mRNA turnover probably represents an active and specific RNA degradation mechanism since many non-cortical granule protein's mRNAs are stable during oocyte maturation (Davidson, 1986). The mechanism of mRNA turnover must also be rapid because oocyte maturation has been shown to be complete within 8.5–16 hours *in vitro,* depending on the species (Grainger *et al.,* 1986; Berg and Wessel, 1997). Taken together, efficiency of this mRNA accumulation and degradation has led to the hypothesis that a similar mechanism can both

activate and suppress the expression of the cortical granule genes during oogenesis, perhaps through genomic positional effects. The rapid pace of cloning sea urchin cortical granule components has constructed a solid foundation to begin looking at the regulatory components common to this unique family of proteins.

## B. Mouse Zona Pellucida

Like vitelline layer components of the sea urchin, zona pellucida glycoproteins are synthesized and secreted by growing oocytes throughout oogenesis. Secretion of the ZP proteins declines near the end of oocyte maturation, and is completely absent upon release of the unfertilized egg at ovulation (reviewed in Wassarman *et al.,* 1985). Reflecting this wave of ZP protein expression is the transcription of Zp genes: At maximum abundance, the ZP transcripts represent 1.5% of the total poly(A)$^+$ RNA (Epifano *et al.,* 1995), but are not detectable following oocyte maturation. Disrupting expression of the genes that encode the zona pellucida genes has also proven to be informative. For example, Zp3 knockout females make mature oocytes that are indistinguishable from wild-type in every way, except that they lack a zona pellucida (Liu *et al.,* 1996; Rankin *et al.,* 1996). These oocytes lacking a zona pellucida are normally infertile, but rescue of fertility experimentally allows the resultant embryos to develop, again, indistinguishable from wild-type embryos. Such gene-inactivation experiments, and a host of others (Rankin and Dean, 2000), show that coexpression of *all* the components of the zona pellucida are crucial for its proper function during fertilization, and that their role in mammals is exclusive to fertilization.

The intimate relationship between mouse cortical granule proteins and the zona pellucida during the block to polyspermy reaction suggests that, like the structural and modifying components in sea urchins, gene expression of these two classes of proteins parallel one another. Pierce *et al.* (1992) was able to trace the expression of a mouse cortical granule protein, p75, throughout oogenesis. By *in vitro* translation, followed by immunoprecipitation with an antibody that recognized a 75-kDa cortical granule protein, they were able to detect minor levels of p75 mRNA in 20-$\mu$m oocytes, the smallest oocytes assayed, and saw a several-fold rise in p75 mRNA levels during development to a full-grown oocyte. Following germinal vesicle breakdown, a marker for oocyte maturation, the p75 mRNA levels plummeted over 90% from peak levels. Taken together, this similarity to the transcription profiles of mouse ZP and sea urchin cortical granules proteins provides compelling evidence that each organism uses similar strategies to regulate gene expression of essential proteins involved in fertilization.

Promoter analysis of the ZP genes has been successful in defining DNA fragments that support their oocyte-specific gene expression. Little sequence similarity exists among the promoters, except for a canonical E-box (CANNTG) located approximately 200 base pairs upstream of the transcription start site of each mouse

ZP gene (Epifano *et al.,* 1995). This conserved *cis* regulatory element is a possible binding site for the tissue-specific basic helix–loop–helix (bHLH) transcription factors. Mutagenesis of the E-box in mouse ZP gene promoters markedly reduce their gene activity in growing oocytes, demonstrating the importance of the E-box in ZP gene expression (Liang and Dean, 1993; Millar *et al.,* 1991).

Recently, a novel germ-cell-specific bHLH transcription factor, FIG$\alpha$ (factor in the germline alpha), has been identified. It accumulates selectively in oocytes within the ovary, binds the E-box of all three mouse ZP genes, and can transactivate reporter genes coupled to each of the ZP promoters *in vitro* (Liang *et al.,* 1997). However, FIG$\alpha$ is not sufficient to activate endogenous ZP gene expression, suggesting that it plays a role in the coordinate expression of the three ZP genes during oogenesis by presumably interacting with other necessary factors. E-boxes are also located on each of the three human ZP genes, and they show similar *in vitro* activity to the mouse ZP genes, suggesting that the role of FIG$\alpha$ may be conserved among mammals, if not throughout the animal kingdom.

## VI. Biogenesis of Cortical Granules and Their Contents

In addition to the postulated functions of cortical granule components following exocytosis, one or more of these may function during cortical granule biogenesis, specifically involved in selective packaging of other proteins. This packaging phenomenon may be particularly complex since each cortical granule component is packaged at a time when a great deal of protein traffic for other secretory vesicles gridlocks the Golgi apparatus. Simultaneously, there is the acquisition of endocytic proteins and macromolecules by vesicles, creating additional traffic within the cytoplasm. How the oocyte keeps these events organized is of particular interest and will be dealt with in the following section.

### A. Organelle Biogenesis

The developing oocyte synthesizes and stores a large mass and diversity of proteins. Most of the proteins and other macromolecules found in developing oocytes are made by the oocyte, but a large quantity of molecules—including yolk and glycogen—are heterosynthetic, contributed by the accessory cells, gut, or liver. Thus, the pathways responsible for organizing membrane-bound proteins in an oocyte are complicated by the influx of proteins, the diversity of molecules, and the timing of regulated trafficking and secretion of these proteins.

Cortical granule biogenesis in these cells occurs concurrently with the biogenesis of several other major types of secretory vesicles. Yet, each type of vesicle contains a unique population of proteins, is translocated to different regions, and is

secreted at distinct times either before, during, or following fertilization. Vesicles types unique to the oocyte include:(1) those containing vitelline layer components (or zona pellucida components in mammals) that are both translocated to the plasma membrane and secreted late in oogenesis;(2) cortical granules that are translocated to the plasma membrane late in oogenesis but secreted at fertilization; and (3) the secretory vesicles containing extracellular matrix molecules that are not translocated nor secreted until several hours following fertilization. Each of these representative secretory vesicles is morphologically and biochemically distinct, yet each is made coincidentally during oogenesis. Thus, each vesicle must have select targeting signals for its contents, for its translocation to the cell surface, and for its secretion. The complexity of biogenesis and the specificity of protein packaging is further increased by the uptake and storage of proteins by the oocyte that are made or transported through somatic cells of the ovary (e.g., follicle cells, accessory cells). Compartmentalization of proteins into different vesicle types is important both for the continued development of the oocyte, for fertilization of the mature egg, and for embryogenesis, when many proteins are either secreted (extracellular matrix proteins and growth factors, for example) or added to the plasma membrane (Alliegro *et al.,* 1992). Cortical granules thus serve as a good model for organelle biogenesis in this cell type because of their abundance, their content diversity, and their distinct morphology.

Cortical granules of mice and sea urchins (the only species for which rigorous data are available) are made continuously through oogenesis. First detected in oocytes 10–15 $\mu$m in diameter, cortical granules accumulate linearly in oocyte development, reaching a maximum population at oocyte maturation of approximately 8000 in mice (Ducibella *et al.,* 1994) and 15,000 in sea urchins (Laidlaw and Wessel, 1994). Biogenesis of cortical granules includes a complete repertoire of components, with no content heterogeneity found between individual vesicles (Hylander and Summers, 1982; Decker *et al.,* 1988; Laidlaw and Wessel, 1994; Wessel, 1989; see, however, Anstrom *et al.,* 1988).

Although descriptions of developing oocytes in sea urchins are extensive, particularly the ultrastructural morphology of organelles in eggs and oocytes (Anderson, 1968; Bal, 1970; Chatlynne, 1969; Verhey and Moyer, 1967), the timing of each oogenic stage is poorly understood and a precise terminology for staging developing oocytes is lacking. Many investigators simply refer to the diameter of the oocytes when describing oocyte stages. The stem cell precursor of oocytes is the oogonia. These cells are approximately 12–16 $\mu$m in diameter and do not have cortical granules. However, oocytes, the immediate progeny of the stem cells, begin to accumulate cortical granules before any substantial growth and differentiation: granules are detectable in oocytes that are less than 20 $\mu$m in diameter (Chatlynne, 1969; Laidlaw and Wessel, 1994). These cortical granules are scattered throughout the cytoplasm and are readily observable in thin sections by electron microscopy (Chatlynne, 1969; Wessel, 1989) or by immunolabeling with antibodies to cortical granule contents (Laidlaw and Wessel, 1994; Wessel, 1989).

Yolk accumulates later in oogenesis when the size of the oocyte increases significantly, from approximately 40 to 70–100 $\mu$m in diameter, depending on the species. Cortical granules continue to accumulate during mid-vitellogenic stages, to approximately 4000–5000 organelles occupying roughly 4500 $\mu m^3$ of space in the oocyte. The volume of cortical granules in mid-vitellogenic stages exceeds the volume of all other organelles in the developing oocyte except for the nucleus (Verhey and Moyer, 1967). By the late germinal vesicle stage, roughly 15,000–18,000 cortical granules are present. During meiotic maturation of the oocyte, the cortical granules then translocate to the periphery of the cell where they attach to the cytoplasmic face of the plasma membrane, and remain poised until fertilization (Fig. 10).

Aside from the observations of cortical granule appearance, the mechanisms of their biogenesis is not understood. When looking at the biology of this organelle, several steps must be considered, and each probably requires the function of select membrane proteins. The mechanisms of protein sorting in the *trans*-Golgi network, for example, may include a two-step sorting process as proposed for the biogenesis of regulated secretory vesicles of somatic cells (Tooze and Stinchcombe, 1992). First, an aggregation-mediated step may initially sort the contents destined for cortical granules from products destined for other organelles. Then a receptor-mediated process may target the aggregate to the appropriate, nascent vesicle that will become a cortical granule. Granule translocation to the cortex presumably utilizes an active, cytoskeletal-mediated process, though this mechanism, in addition to the docking and secretion steps, is not known. Attachment of granules to the cytoplasmic face of the plasma membrane is inherently specific since the cortical granules adhere to only one membrane type among many possibilities. This attachment is also very strong since it can withstand centrifugal forces that displace other organelles of the cell (Harvery, 1956; Hylander and Summers, 1981) and hold up to the disruptive forces of cell homogenization. This property has been used to isolate cortical granules, either as a cell surface complex of cortical granules and the plasma membrane in bulk (Detering *et al.,* 1977; Kinsey, 1986) or as a lawn of cortical granules attached to the plasma membrane (Vacquier, 1975b). The ease of isolation and functionality of such lawns or complexes is frequently used for *in vitro* studies of exocytosis (reviewed in Whitaker (1994) and egg activation (Belton and Foltz, 1995; Moore and Kinsey, 1994).

## B. Protein Sorting and Targeting

The complexity of the secretory pathway in oocytes, both in protein packaging and in timing of translocation to the cell surface for secretion, suggests that multiple mechanisms of packaging are used. In cortical granules alone, at least 12 different proteins are packaged into distinct subcompartments of the same vesicle. When

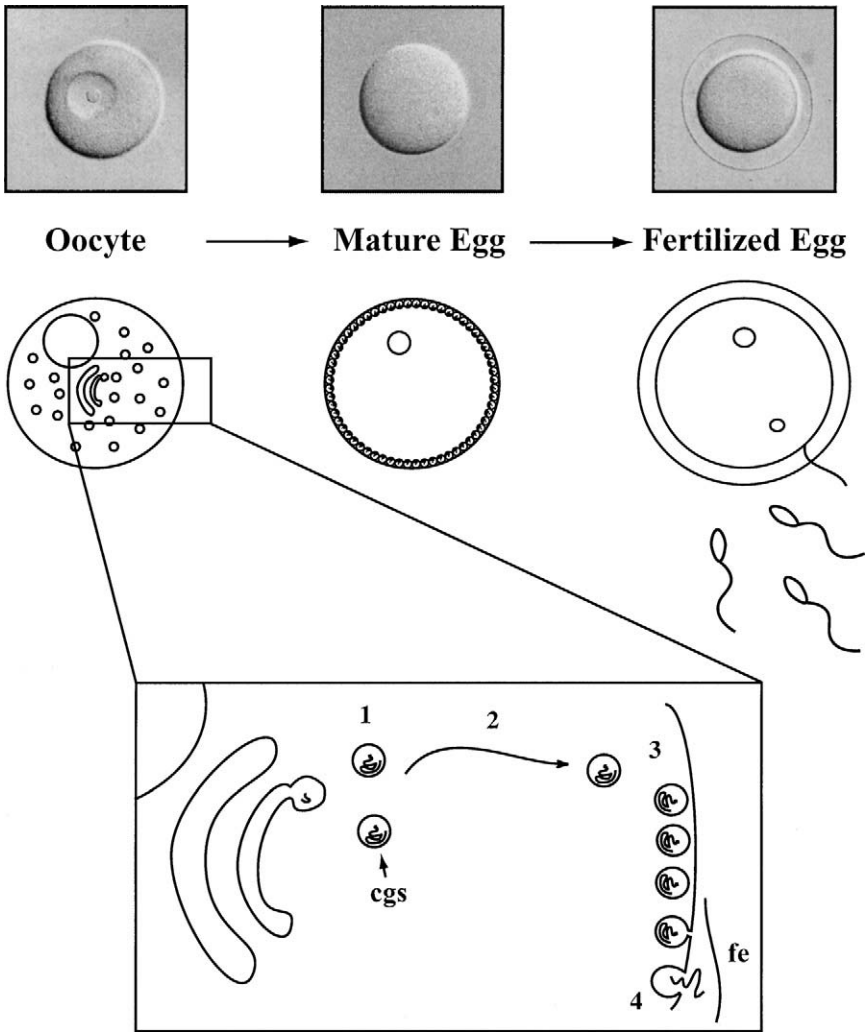



FIG. 10 The biology of cortical granules in the sea urchin. (1) Cortical granules are produced and accumulate in the developing oocyte. (2) Late in oogenesis, cortical granules translocate *en masse* to the cortex and (3) dock with the plasma membrane where, upon sperm stimulation, they (4) fuse and exocytose their contents, giving rise to the permanent block to polyspermy, the fertilization envelope. cgs, cortical granules; fe fertilization envelope.

generating models to explain oocyte vesicle packaging, we must eliminate those based on temporal or spatial specificity, since several different, nonoverlapping secretory vesicles are made at the same time and location within the oocyte. We can also eliminate packaging based strictly on a default secretory mechanism for the same reason. Instead, we favor a mechanism of active and directed packaging into distinct vesicles. This mechanism must integrate many different proteins into the same vesicle, be continuously active throughout oogenesis, and must allow for heterogeneity in the timing of vesicle transport and secretion.

The biogenesis of mature secretory granules is preceded by formation of immature secretory granules from the *trans*-Golgi network (TGN). A characteristic feature of this pathway is the aggregation and condensation of secretory proteins leading to the formation of a distinct dense-core vesicle material. It is not known whether protein aggregation occurs before or after targeting to putative sorting receptors in the TGN. The segregation of distinct proteins in the lumen of the TGN could occur by clustering of receptors in distinct regions of the TGN or by the tendency of regulated proteins to first form aggregates in the TGN which exclude other proteins, followed by receptor binding (reviewed in Chidgey, 1993).

How cells concentrate vesicle material is another unanswered question. Some targeting signals have been identified that direct proteins to a particular intracellular location. This often occurs by interaction with a specific receptor. Receptors in the TGN have different specificities for individual regulated proteins to cluster in distinct regions of the TGN. One of the best-characterized examples is the targeting of lysosomal hydrolases to lysosomes. These hydrolases acquire mannose-6-phosphate residues during passage through the Golgi, allowing the enzyme to be recognized by a transmembrane mannose-6-phosphate receptor in the TGN (Kornfeld, 1987; Punnonen *et al.,* 1996).

In a series of electron micrographic studies utilizing several different species of sea urchin, Everett Anderson (Anderson, 1968, 1974) documented the association between developing cortical granules and the Golgi. He concluded from these studies that the cortical granules originate from an aggregation of precursor vesicles emanating from the Golgi. Although the exact route of component biosynthesis has not been formally proven, it is generally accepted that most cortical granule constituents transit the Golgi before entering the cortical granule. Also noted in these studies was that the Golgi synthesized large amounts of yolk and, more recently, many other classes of secretory vesicles (Alliegro *et al.,* 1992).

Concurrent with cortical granule biogenesis, is the formation of several other classes of secretory vesicles. Each of these secretory vesicles harbors different contents, has a distinct morphology, and is secreted at different times, before, during, or after fertilization. The complexity of vesicle secretion appears far greater following fertilization than during oogenesis. Yet during oogenesis, the secretory

pathway is complicated by the continuous endocytosis of yolk and glycogen.[2] Vesicles harboring the glycoproteins for construction of the vitelline layer and the jelly coat secrete their contents before fertilization, especially during the middle to late stages of oogenesis (Verhey and Moyer, 1967). Translocation of the cortical granules to the cortex does not occur until late in oogenesis, probably since the exocytosis and endocytosis of other vesicles is occuring in the developing oocyte.

Other vesicles made during oogenesis are also stored for prolonged periods and are not secreted until several hours following fertilization. Oocytes have been shown to package echinonectin (Alliegro *et al.,* 1992, 1988), fibropellins (Bisgrove *et al.,* 1991; Bisgrove and Raff, 1993), other apical lamina proteins (Alliegro and Schuel, 1988), and a variety of molecules destined for the extracellular matrix (Berg *et al.,* 1996; Wessel *et al.,* 1984) as well as proteins destined for the cell surface (Nelson and McClay, 1988). Each of these proteins appears to be packaged into distinguishable vesicles, each of a distinct relative density due to its unique assortment of constituents, and is secreted at different times following fertilization. This diversity in oocytes and eggs suggests a complexity in the secretory pathway not seen in somatic cells.

In the sea urchin ovary, cortical granules are also seen in the somatic-derived accessory cells. These cortical granules, however, are packaged within larger structures of approximately 10–15 $\mu$m in diameter, known as mosaic granules or mosaic globules, the term deriving from their heterochromatic histological staining (Masuda and Dan, 1977). The cortical granules of mosaic granules are indistinguishable from those of developing or mature eggs. Morphologically, each cortical granule of a mosaic granule is of similar size and contains an internal pattern identical to that seen in oocytes. Moleculary, cortical granules from each of the cell types harbors the same epitopes, each at the same relative level of labeling. Yet the cyclic abundance of mosaic granules suggests that they are a remnant organelle resulting from the phagocytosis and degradation of atretic oocytes (Chatlynne, 1969). This conclusion is supported by evidence that the accessory cells do not accumulate detectable levels of mRNA encoding cortical granule proteins (Laidlaw and Wessel, 1994) and by the observation of yolk and glycogen dispersed within mosaic granules. It is not clear why or how cortical granules—as opposed to yolk or other secretory vesicles—are so stable in the accessory cells, present months after any mature eggs are visible, but it might provide insight to the final stages of cortical granule biogenesis.

[2] Yolk is made by both the oocyte and the adult intestines. The yolk made by the adult is transported to the ovary, where it passes through the double basal lamina and muscular ovarian capsule, through the accessory cells, and into the developing oocytes. The mechanisms involved in this pathway are unknown. Curiously, males also make yolk in their intestines and testis (Shyu *et al.,* 1986), so perhaps the major yolk protein is a universal energy currency for gamete development. Significant storage, though, of the major yolk protein occurs only in the oocytes.

## VII. Translocation

### A. Translocation of Cortical Granules to the Plasma Membrane

Cortical granules accumulate and are randomly distributed within the cytoplasm during oogenesis in most animals. At some point during maturation, these granules translocate to the oocyte surface and anchor to the plasma membrane, which is a prerequisite for exocytosis. Timing, patterns, and regulation of cortical granule translocation appear to vary in different species. For example, in sea urchin, cortical granules translocate from the oocyte cytoplasm to the cell surface *en masse* just prior to oocyte maturation. In other species, such as starfish, cortical granules move to the cortex and accumulate there throughout oogenesis, whereas in mice, the cortical granules translocate during meiotic maturation but do not dock at the plasma membrane until after fertilization. Cortical granules are morphologically and biochemically different from all other secretory vesicles of the oocyte (Anderson, 1974; Shapiro *et al.,* 1989), and their translocation appears to be organelle-specific since cortical granules are the only organelles known to accumulate at the oocyte surface. Although the regulation of cortical granule translocation has been addressed only in a few species, significant progress has been made in identifying its mechanism. Of particular note is the mounting evidence in these model organisms that actin microfilaments are critical in cortical granule translocation.

### B. Invertebrates

#### 1. Sea Urchin

In the sea urchin, cortical granules accumulate randomly throughout the cytoplasm during oogenesis, but in mature eggs they are attached to the plasma membrane, having translocated to the cortex at some earlier time. Once at the surface, cortical granules adhere selectively and tightly to the plasma membrane; they cannot be dislodged by standard homogenization nor centrifugation (Vacquier, 1975b), although hydrostatic pressure and certain anesthetics have been shown to separate them (see above; Decker and Kinsey, 1983; Hylander and Summers, 1981; Longo and Anderson, 1970).

The process of cortical granule translocation in *in vitro*-matured oocytes begins with the movement of the germinal vesicle to the oocyte cell surface, and is 50% complete once germinal vesicle breakdown (GVBD) has occurred. In the *in vitro*-matured egg, 99% of the cortical granules can be found at the cortex, a phenotype indistinguishable from translocation that occurs in oocytes that mature *in vivo* (Berg and Wessel, 1997). The translocation event appears to be very rapid, particulary since intermediates of cortical granule movement are seldom seen. By the time the

pronucleus is apparent in these cells, 99% of cortical granules have translocated. Thus, the machinery for cortical granule translocation is initiated early in meiotic maturation and persists until late in the maturation process.

## 2. Starfish

Cortical granules of starfish oocytes progressively translocate to the oocyte surface during individual granule biogenesis. Thus, in immature oocytes, cortical granules are dispersed throughout the cytoplasm (Reimer and Crawford, 1995). Upon maturation of the oocyte, most of the granules can be found at the cell cortex, but a few granules remain in the central cytoplasmic region. Contrary to what is normally seen, it has been reported that some cortical granules remain in the cytoplasm following fertilization (Reimer and Crawford, 1995). The suggested rationale for this delay is that two waves of exocytosis occurs: one during fertilization and another during embryogenesis, at least until completion of second cleavage. The consequences of this delay in exocytosis have not been indicated, although we may speculate that certain cortical granule proteins could serve a protective function in later stages of development.

In starfish oocytes, treatment with 1-methyl adenine (1-MA) stimulates the progression through meiosis as well as cortical granule translocation toward the oocyte surface. In species with oblong cortical granules, it was noted that they even orient themselves with their long axes perpendicular to the oocyte surface (Longo *et al.,* 1995). An unusual rise in intracellular calcium is induced with 1-MA treatment, suggesting that this may be responsible for at least some of the morphological changes observed during the maturation process. Injection of heparin, a functional blocker of IP3 receptors on the endoplasmic reticulum, abolished 1-MA-induced cortical granule translocation as well as the rise in cytoplasmic calcium levels (Santella *et al.,* 1999). Thus, in starfish oocytes translocation is, in part, a result of an IP3-mediated calcium release—a process reminiscent of the hormone-induced translocation of pancreatic beta cell granules by an actin–myosin cytoskeletal rearrangement (Fig. 11; Niwa *et al.,* 1998).

Closer examination of the actin cytoskeletal structure of these heparin-injected, 1-MA-stimulated oocytes revealed extensive cytoplasmic bundles and a generally disrupted organization in the egg cortex. Treatment with cytochalasin B to disrupt microfilaments resulted in a clustering of cortical granules and a failure to orient perpendicularly to the plasma membrane. Taken together, these inhibition studies strongly indicate that the process of cortical granule translocation is dependent on actin rearrangement in starfish oocytes (Santella *et al.,* 1999). Whether the actin–myosin model of calcium-regulated cortical granule translocation extends to other organisms is speculative, but extremely interesting considering that this movement does not follow the traditional dogma for actin-mediated cytoplasmic reorganization.

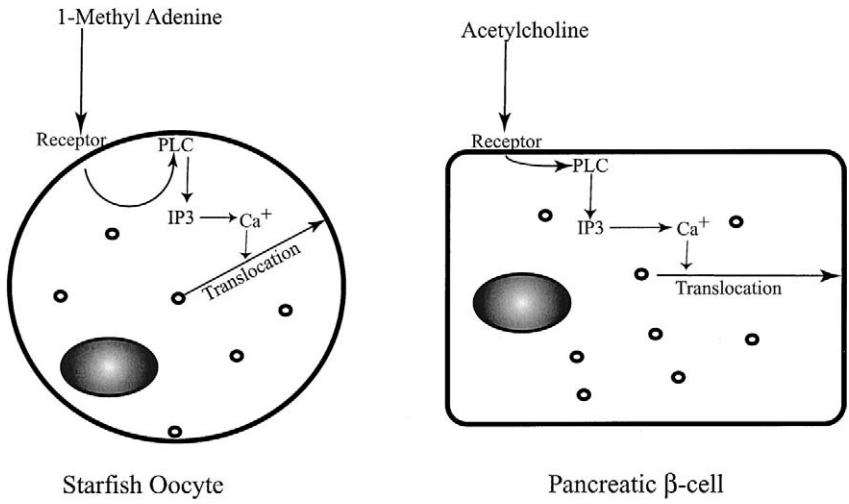



FIG. 11 Phospholipase C (PLC) is activated upon receptor activation by the hormonal stimulus. It leads to the increase in cytoplasmic calcium; which in turn (in pancreatic beta-cells) activates myosin light chain kinase, resulting in phosphorylation of myosin light chain and translocation of secretory granules. IP3, inositol trisphosphate.

## C. Vertebrates

### 1. Fish

In fish, cortical granules (also referred to as "yolk vesicles," "intravesicular yolk," and "endogenous yolk") initially appear circumferentially and, depending on the species, may occupy many focal shells within the cytoplasm. By the time vitellogenesis commences, cortical granules of varying maturity occupy most of the oocyte cytoplasm almost entirely, and they continue to form and translocate to the periphery of the oocyte in subsequent stages of oogenesis. The current hypothesis suggests that the cortical granules are displaced to the plasma membrane by centripetal accumulation of yolk proteins. Once oocyte maturation has completed, all cortical granules can be found docked beneath the plasma membrane, at which time they are referred to as "cortical alveoli" (reviewed by Wallace and Selman, 1990). *Danio rerio* eggs show a characteristic, size-dependent cortical granule distribution (Becker and Hart, 1999): smaller cortical granules form a relatively uniform population of vesicles immediately beneath the plasma membrane while larger cortical granules are encountered at deeper focal planes of the cortex.

### 2. Amphibians

Cortical granules of amphibian eggs begin to form when vitellogenesis is initiated in the outer third of the oocyte cytoplasm. In the subsequent stages of oogenesis, the

granules may be found distributed throughout the cytoplasm and in some species such as *Rana pipens*, they increase in size and number as the oocyte grows (Kemp and Istock, 1967). In the full-grown, stage IV amphibian oocyte, cortical granule aggregates are present in submembrane regions (Dabike and Preller, 1999; Grey *et al.,* 1974; also reviewed by Guraya, 1982). Grey *et al.* (1974) have described two kinds of cortical granules in *Xenopus laevis* eggs: small granules are localized in the animal half of the oocyte where they are aligned in a single layer, whereas large granules accumulate on the vegetal half of the egg and usually occupy two to three layers (Balinsky, 1966; Grey *et al.,* 1974).

During the final stages of maturation, these cortical granules achieve a more uniform arrangement directly beneath the egg surface (Larabell and Chandler, 1988a). The method of reorganization has been addressed on a minor level and appears to involve actin. Treating *Xenopus* GV-stage oocytes (a stage where cortical granules are already positioned at the surface) with cytochalasin B failed to displace cortical granules from the cortex (Ryabova *et al.,* 1986), whereas yolk and pigment granules were readily displaced from discreet zones of the oocyte surface. However, the same treatment of metaphase-II-arrested eggs did induce disturbance in cortical granule localization: in some regions, cortical granules formed large clusters, while in controls they were absent, even though there was no apparent displacement of cortical granules to the interior of the egg. This shows that translocation events during later stages of maturation are dependent on actin to move along the classic paths, but once the granules are positioned within the oocyte cortex the process is no longer dependent on the actin cytoskeletal. The latter event may be a result of the proteins involved in docking the vesicles to the plasma membrane (see below).

## D. Mammals

Mature cortical granules of mammalian oocytes can first be identified at the periphery of the *trans*-Golgi complex. Following separation from the Golgi stacks, these free granules either migrate toward the surface of the oocyte or gather into small clusters. Only a few cortical granules are found adjacent to the plasma membrane in preovulatory bovine, hamster, human, ovine, and rabbit follicular oocytes (Baca and Zamboni, 1967; Fleming and Saacke, 1972; Szollosi, 1967); most of the granules gather in clusters or disperse as individuals in the subcortical cytoplasm (reviewed by Gulyas, 1979).

Upon maturation of the oocyte, the majority of cortical granules can be found within a 2-$\mu$m layer at the periphery of the egg, although some granules have been seen to persist in deeper regions of the ooplasm. Electron microscopy of these granules reveals a range of characteristics in these cortical granules, from uniform, as found in rabbit (Krauskopf, 1968a; Krauskopf, 1968b), human (Baca and Zamboni, 1967), and monkey (Hope, 1965), to variably dark or light or mosaic, as seen in mouse, hamster, rat or porcine eggs (Cran *et al.,* 1988; Nicosia and

Mikhail, 1975; Raz *et al.,* 1998a,b; Selman and Anderson, 1975; Szollosi, 1967). Considering that both light and dark cortical granules can be found adjacent to the plasma membrane of the mature egg, it has been proposed that these different types represent states of cortical granule content maturation, or a functional heterogeneity within the granule population (Gulyas, 1979).

The timing and distribution of cortical granules during mammalian oogenesis may not depend on innate timing of intracellular events. Rather, corona cells surrounding the developing oocyte may regulate the synchronicity of events (Szollosi, 1978). For example, not all follicle-enclosed mammalian oocytes undergo spontaneous meiotic maturation when cultured *in vitro*. If, however, these cultured oocytes are exposed to gonadotropins such as lutienizing hormone (LH), follicle stimulating hormone (FSH), or human chorionic gonadotropin (hCG), or if they are removed from the follicle, they will resume meiosis (Nicosia *et al.,* 1977; Szollosi, 1967). These observations led to the suggestion that the corona cells exert an inhibitory effect on oocyte maturation and subsequently on cortical granule translocation (Szollosi, 1978). Not until the effect was broken by hormones would the oocyte continue through meiosis II. Of course, there are the exceptions, which will be indicated for each relevant organism that follows.

## 1. Bovine

Cortical granule accumulation and translocation in bovine oocytes falls into three general types, each describing a different morphological stage within the cytoplasm: type I represents predominantly aggregated cortical granules (5–10 cortical granule aggregates and less than 50 dispersed particles per 1000 $\mu m^2$ of the cortex) throughout the cytoplasm; type II indicates the beginning of cortical granule dispersal beneath the plasma membrane (oocytes with 1–4 cortical granule aggregates and more than 50 dispersed particles per 1000 $\mu m^2$ of the cortex); and type III describes a mature cortical granule distribution (Fig. 12; completely dispersed cortical granules that are distributed in the cortex and form a monolayer when observed in the equator of the oocytes; the number of cortical granules range from 224 to 348 per 1000 $\mu m^2$ of the cortex) (Wang *et al.,* 1997). Generally, immature oocytes in the GV stage show a type I distribution pattern of cortical granules, partially matured metaphase-I oocytes exhibit a type II pattern, and fully matured metaphase-II oocytes show a type III pattern (see figure). The tendency of cortical granules to aggregate in oocytes containing germinal vesicles or arrested in metaphase I is a phenomenon very similar to those reported in anuran oocytes (Guraya, 1982). As in anuran oocytes, further maturation of bovine oocytes corresponds with a centrifugal dispersal of the clustered cortical granules, resulting in a single layer of individual granules lying directly beneath the plasma membrane.

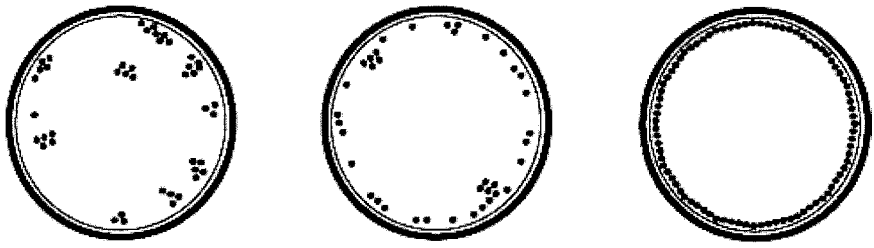



FIG. 12 Three general phases of cortical granule accumulation and translocation in bovine oocytes.

Comparison of cortical granule translocation in either *in vitro*- or *in vivo*-matured, cumulus oopherus-enclosed bovine oocytes shows that the majority of *in vitro*-matured oocytes exhibit delays in cortical granule migration and dispersal (displaying mostly type I and type II cortical granule distribution) compared to *in vivo* matured controls: *In vitro* oocyte populations display mostly type I and type II granule distribution, whereas 95% of *in vivo* populations of GV stage oocytes arrested at metaphase-I show type I cortical granule distribution with the remaining 5% showing type II distribution. When the oocytes were allowed to proceed to metaphase-II arrest *in vitro*, the proportions of oocytes with type I cortical granule distribution decreased to 25%. Accordingly, the proportions of oocytes with type II and type III cortical granule distribution increased to 42% and 33%, respectively (Wang *et al.,* 1997). The absence of coordination between the nuclear and cytoplasmic maturation of the oocytes probably suggests differences in the regulatory mechanisms responsible for their individual progression.

Alternatively, these two processes may be controlled by different hormone-mediated signals from the cumulus cells of the oocyte follicle. *In vivo*, growth hormone (GH) is synthesized in antral bovine follicles and is thought to exert paracrine and/or autocrine action in follicle growth and oocyte maturation (Izadyar *et al.,* 1999). Exposure of cumulus-enclosed bovine oocytes in *in vitro* culture to GH accelerates nuclear maturation, and promotes subsequent cleavage and embryonic development (Izadyar *et al.,* 1998). In fact, GH supplementation during *in vitro* maturation significantly enhanced migration and dispersal of cortical granules such that after 22 h of *in vitro* maturation in the presence of GH, metaphase-II oocytes showed a significantly higher type III cortical granule distribution than those that matured in the absence of GH (48% as compared to 24%) (Izadyar *et al.,* 1998). Thus, when *in vitro* maturation proceeds in the presence of GH, a coordination between the nuclear stage of the oocyte and the localization of cortical granules can be seen that was not observed before, strongly suggesting that bovine oogenesis is tightly regulated by neuroendocrine hormones.

## 2. Porcine

In contrast to the bovine system, germinal vesicle-stage porcine oocytes contain a dispersed array of cortical granules within 6–10 $\mu$m of the cell perimeter, usually at a density of 33.8 $\pm$ 7.3 granules per 100 $\mu m^2$. Progression through GVBD to metaphase I initiates the mass migration of all cortical granules toward the plasma membrane such that they end up in a monolayer at maturation. Cortical granule density at this stage increases to mean values of 45.3 $\pm$ 4.5 granules per 100 $\mu m^2$ at metaphase-I and 45.7 $\pm$ 6.2 granules per 100 $\mu m^2$ at metaphase-II. Unlike organisms previously described, this metaphase-I translocation results in the loss of cortical granules to the first polar body (Wang *et al.,* 1997).

Another difference found in porcine oogenesis is the lack of cortical granule translocation during *in vitro* culturing. After 46 h of culturing, GV-stage oocyte populations display up to 90% progression beyond the GVBD stage, but none of these cells has indicated any differences in cortical granule distribution. Therefore, it is hypothesized that cortical granule translocation is somehow coordinated with meiotic maturation.

As in bovine, the key to this developmental process may lie with the influence cumulus cells have on porcine oocytes. When porcine oocytes are denuded at 5 or 20 h of culture, and further cultured *in vitro* without cumulus cells, they showed a higher percentage of cortical granules "in contact" with plasma membrane than "near" it at maturation (75% vs 24%). In contrast, when cumulus cells were removed after 30 h of culture, or were left for the whole maturation period, the oocytes showed equal numbers of granules "in contact" and "near" plasma membrane (Galeati *et al.,* 1991). This strongly suggests that prolonged exposure to a cumulus cell signaling factor is essential for the final steps of oocyte maturation. The cellular effects of this factor, however, have yet to be elucidated.

## 3. Rodents

During mouse oocyte growth, the cortical granules increase in number and migrate toward the cortex, assuming a final position within 2 $\mu$m of the plasma membrane (Ducibella *et al.,* 1988a). From the primary oogonia to the full grown oocyte, cortical granules accumulate to a final count of $\sim$6000–8000, at a cortical density of 14 granules per 100 $\mu m^2$ (Ducibella *et al.,* 1994). Although smaller oocytes have a lower mean cortical granule density than GV-stage oocytes, they appear to be very dense due to their tendency to cluster. Completion of cortical granule maturation in full-grown GV- stage oocytes is accompanied by a dispersal of individual granules to their final position just below the plasma membrane (Ducibella *et al.,* 1988a,b).

While the majority of the cortical granules are peripherally localized at the end of oocyte growth, some cortical granules have been found to remain in the interior of the oocyte. Oocyte maturation is accompanied by the translocation of these late granules, as well as the formation of a cortical granule-free domain

(CGFD) overlying the metaphase II spindle (Ducibella *et al.,* 1988b). The CGFD, which is observed exclusively in rodents, is a result of partial exocytosis of cortical granules in the vicinity of the meiotic spindle. As a consequence of this maturation-associated cortical granule release, the metaphase-II arrested egg contains merely ~4000 cortical granules as compared to the original 6000–8000 in full-grown oocytes. Coincident with the formation of the CGFD is the generation of an microvillar-free domain and cortical actin thickening over the metaphase spindle (Longo, 1985), as well as an increase in the apparent cortical granule density (Ducibella *et al.,* 1988b).

Changes in the distribution of cortical granules appear to be tightly linked to the process of maturation since blocking oocyte maturation in the presence of the inhibitor dbcAMP also blocks translocation (Connors *et al.,* 1998). Further characterization of this process utilized inhibitors of both microtubule and actin dynamics to address the players involved in the cytoplasmic maturation versus cortical granule translocation steps. Treatment of eggs with the microtubule inhibitors nocodazole or colchicine specifically inhibited the formation of a CGFD over metaphase-II spindles. Rather, the classic scattering of chromosomes was emphasized by a smaller CGFD over each individual chromosome, suggesting that the chromosome or chromosome-associated material, and not the spindle *per se* (Ducibella *et al.,* 1990), dictates the localization of the CGFD (Connors *et al.,* 1998). Following maturation of these treated oocytes, all cortical granules were still localized at the egg cortex (Connors *et al.,* 1998). Therefore, it was concluded that cortical granule movement does not involve microtubules.

Treatment of mouse metaphase-II-arrested eggs with cytochalasin D, which disrupts filamentous actin, results in a retention of cortical granules in the interior of the egg. Similarly, treatment with cytochalasin B, another disrupter of filamentous actin, did not dramatically change the position of the cortical granules, although there was more disorganization within the monolayer (Connors *et al.,* 1998). Together, these cytochalasin studies suggest that once located at the cortex, a mechanical block prevents retrograde movement of the cortical granules back toward the Golgi. The possibility that both microtubules and microfilaments are involved in, or could compensate for each other in, maintaining cortical granules at the cortex is excluded by simultaneous treatment of metaphase-II-arrested eggs with both cytochalasin D and nocodazole or colchicine. The cortical granules in such treated eggs remain at the cortex (Connors *et al.,* 1998).

Unlike other mammalian systems studied, *in vitro* matured, cumulus-denuded mouse oocytes exhibit wild-type cortical granule distribution and density, as assayed by electron, fluorescent, and confocal microscopy following *Lens culinaris* agglutin labeling (Cecconi *et al.,* 1998). Thus, interruption of the functional interaction between cumulus cells and oocyte does not appear to affect the mouse oocyte's capacity to normally synthesize and distribute cortical granules, which has important implications for researchers of cortical granule biology as well as for clinicians in *in vitro* fertilization clinics (see below).

## VIII. Regulation of Cortical Granule Docking and Secretion

### A. Cortical Granule Exocytosis

Following translocation to the cortex of the egg, the cortical granules remain poised until stimulated at fertilization to exocytose their contents, which in some animals may be for weeks, or experimentally for months. Cortical granule fusion is a calcium-dependent membrane fusion event that requires an increase in intracellular free calcium. Although the signal transduction pathway leading to calcium release is not completely defined, it is known that sperm binding initiates the release of calcium from endoplasmic reticulum stores in the sea urchin (Foltz, 1995; Terasaki *et al.,* 1991). Within 25 s following egg activation by sperm, cytoplasmic free calcium concentrations reach 1.5–2.5 $\mu$M (Shen, 1995; Whitaker and Swann, 1993), causing the rapid exocytosis of cortical granule contents (Fig. 13). Differential responsiveness to calcium of an egg's cortical granules have been reported, indicating that calcium sensors on individual cortical granules may differ in amount or sensitivity (Matese and McClay, 1998).

Exocytosis of cortical granules begins at the point of sperm/egg fusion and extends in a wave around the perimeter of the egg (Fig. 2). In the mature, fertilizable egg of animals such as the sea urchin, starfish, and frog, cortical granules are attached to the plasma membrance and thus do not require any vesicle transport for subsequent fusion. As a consequence, cortical granule fusion begins within seconds of fertilization. This contrasts the mouse oocyte in which cortical granules must translocate 1–2 $\mu$m to the plama membrane before exocytosis may occur, resulting at least in part to the longer time required to complete cortical granule exocytosis (20–60 mins) (Ducibella, 1996). While neither the mechanism of cortical granule interaction with the plasma membrane nor the calcium sensor for exocytosis is known, recent studies of the sea urchin egg have provided insight into the potential molecular mechanisms of this process, and thus will be the prominent focus of this section.

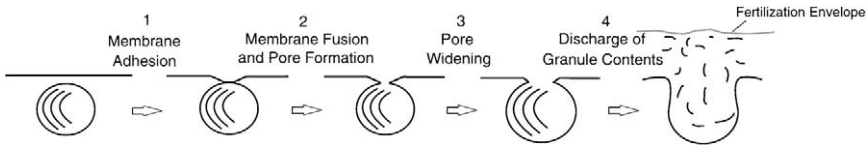



FIG. 13 Diagrammatic summary of the four experimentally separable steps in cortical granule exocytosis (after Chandler *et al.,* 1989). (Reproduced with permission from A protein of the sea urchin cortical granules is targeted to the fertilization envelope and contains an LDL-receptor-like motif. G. M. Wessel, *Developmental Biology,* 1995;167:388–397. Copyright ©1995, Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

The biology of cortical granules in the sea urchin egg provides a unique opportunity to study protein-mediated membrane fusion (Vacquier, 1976). Since cortical granules dock at the cortex of the egg and wait for increases in free calcium levels to exocytose, the processes of cortical granule docking and fusion can be clearly separated experimentally (Fig. 10).

## B. Isolation of Functional Cortical Granule Complexes

The attachment of sea urchin cortical granules to the plasma membrane is firm and resistant to separation by strong shear forces (Vacquier, 1975b). This property has been utilized to isolate cortical granule "lawns," cellular fragments containing the vitelline layer and the egg plasma membrane with attached exocytosis-competent cortical granules. Cortical granule lawns can be isolated in minutes on a polycation-coated Petri dish (Vacquier, 1975b) or *en masse* by homogenization followed by differential centrifugation (Fig. 14; Detering *et al.,* 1977; Kinsey, 1986). Similar techniques have also been devised to isolate cortex preparations from Xenopus (Wall and Patel, 1989). This has provided the ability to isolate cortical granules in large amounts for biochemical studies, and experimental access to both sides of the plasma membrane for *in vitro* investigations. Such *in vitro* systems have provided strong evidence for the calcium-dependency of cortical granule exocytosis by showing that "intracellular" calcium concentrations above 2.75 $\mu$M can induce complete exocytosis of cortical granules in the lawn (Vacquier, 1976).

Cytoskeletal elements do not appear to be important for the fusion reaction since treatment of sea urchin eggs with colchincine, colcemid, or cytochalasin does not influence the fusion rate or the calcium sensitivity (Vacquier, 1976; Whitaker,

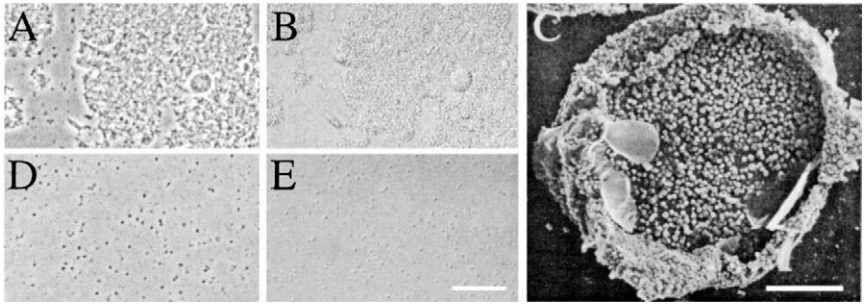



FIG. 14 Cortical granule isolation. Fusion-competent cortical lawns attached to glass slides can be rapidly isolated and easily visualized by either phase-contrast (A) or differential interference contrast (B) microscopy. Alternatively, cell-surface-complex samples, shown here by scanning electron microscopy (C), can be isolated *en masse* by simple homogenization and differential centrifugation. Either cortex preparation can be then used to isolate cortical granules to greater than 95% homogeneity, seen in phase-contrast (D) or differential interference contrast (E). Bar, 10 $\mu$m.

1994) and pretreatment of isolated cortical granules or the plasma membrane with high calcium levels (~1 mM) does not affect the competency of vesicle fusion when reconstituted (Whalley and Whitaker, 1988). This $Ca^{2+}$-mediated release is inhibited both *in vitro* and *in vivo* by local anesthetics such as procaine (Hylander and Summers, 1981) and by exposure to cation mimics lanthanum, cobalt, and manganese (Vacquier, 1975b).

A biochemical characterization of cortical granule membranes has been made possible by isolating large quantities of cortical granules from the cell surface complex. Decker and Kinsey (1983) found that cortical granules may be dissociated from the plasma membrane *in vitro* by using 1 M sucrose. The released cortical granules are then purified from the plasma membrane contaminants using sucrose gradient fractionation. By analyzing the lipid constituents of the plasma membrane and cortical granule membrane, these investigators determined that although the content of cholesterol in cortical granule membranes was three times that of the plasma membrane, the overall fatty acid composition between cortical granules and the plasma membrane was quite similar, with palmitate, stearate, and arachidonate accounting for over 50% of the fatty acids. Thus, looking solely at lipid content, one would not expect a significant change in the overall lipid constitution of the egg plasma membrane when cortical granules exocytose. Wolf *et al.* (1981), however, used fluorescent lipid analogs to distinguish lipid domains in the plasma membrane of the fertilized egg, suggesting that the plasma membrane of the fertilized egg may be a functional mosaic that is important in the massive re-endocytosis of membrane following fertilization (Terasaki, 1995; Whalley *et al.,* 1995; Smith *et al.,* 2000).

Vater and Jackson (1989) took a different approach and identified proteins present selectively in the cortical granule membranes. These investigators separated cortical granules from the plasma membranes using alkaline conditions, and the cortical granule membranes were then further purified by sucrose gradient centrifugation. The membrane protein composition was analyzed by SDS–polyacrylamide gel electrophoresis and found to be distinct from the membrane proteins of yolk platelets and the plasma membrane (Vater and Jackson, 1989). Over a dozen bands were detected in isolated cortical granule membranes, a few of which appeared to be associated peripherally, based on their absence following alkaline extraction. The two major peripheral proteins of this preparation appeared to be $\alpha$-spectrin and actin, but the identity of the other peripheral and integral membrane proteins is yet unknown. The unique membrane–protein composition of the cortical granules, the yolk platelets, and the plasma membranes probably reflects unique functional requirements of each organelle, and may aid in their distinction during biogenesis.

Examination of the egg by quick-freeze fixation, followed by freeze–fracturing, revealed the intramembranous particles of the plasma membrane and the cortical granule membrane (Chandler and Heuser, 1979). This study showed that the intramembranous particles of the cortical granules are dispersed randomly throughout the cortical granule; therefore, no specialized zones or clearings that would

be indicative of a predetermined region where fusion might occur appear to exist morphologically. It also showed that the particle density of cortical granules is significantly less than that of the plasma membrane. Immediately following exocytosis, the membrane of the cortical granules remain distinct from the plasma membrane, suggesting that the membranes, or at least the intramembranous particles, do not readily mix (Chandler and Heuser, 1979).

Calcium-dependent membrane fusion is a specific interaction between cortical granules and the plasma membrane *in vivo*. Unlike other egg vesicles (McNeil *et al.,* 2000), cortical granules do not spuriously fuse with each other, other vesicles, closely adjacent membranes of the endoplasmic reticulum (Terasaki and Jaffe, 1991), or the Golgi apparatus. *In vitro*, however, cortical granule homotypic fusion events are possible (Vacquier, 1975b) and have been used as a model system to study the biophysics of membrane fusion in a defined system (Coorssen *et al.,* 1998; Tahara *et al.,* 1998; Vogel *et al.,* 1991), though superphysiological levels of calcium ($\sim$100 $\mu$M) are required for efficient vesicle–vesicle fusion.

What creates vesicle docking and fusion specificity? It is resonable to hypothesize that the lipid and/or protein composition of the vesicle membrane contains the necessary information for specific targeting, docking, and subsequent fusion during appropriate stimulation. While lipid composition studies comparing membranes of cortical granules with that of the plasma membrane in the sea urchin egg have revealed few overall differences (see Decker and Kinsey, 1983), proteins have been identified that appear to be selective for cortical granule membranes which are not found on the plasma membrane or membranes of yolk platelet vesicles (Vater and Jackson, 1989). Moreover, proteolytic treatment of isolated sea urchin egg cell surface complexes results in a decreased sensitivity of cortical granule fusion to a calcium stimulus (Jackson *et al.,* 1985). Thus, it seems likely that vesicle targeting, docking, and fusion with the plasma membrane are each protein-mediated events. What proteins could be responsible for cortical granule membrane fusion?

## C. Protein Mediated Membrane Fusion

In recent years, two classes of proteins have emerged as playing key roles in protein-mediated membrane fusion (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Rothman and Warren, 1994). The first class of proteins are a group of membrane-associated proteins found on vesicle and target membranes, known collectively as v- and t-SNAREs (soluble NSF-attachment protein [SNAP] receptors), respectively. These proteins spontaneously assemble into a highly stable ternary complex made of the t-SNAREs, syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), and the v-SNARE VAMP (vesicle-associated membrane protein)/synaptobrevin. This complex appears to function as the core machinery necessary for membrane fusion (Fig. 15, Fasshauer *et al.,* 1998a; Weber

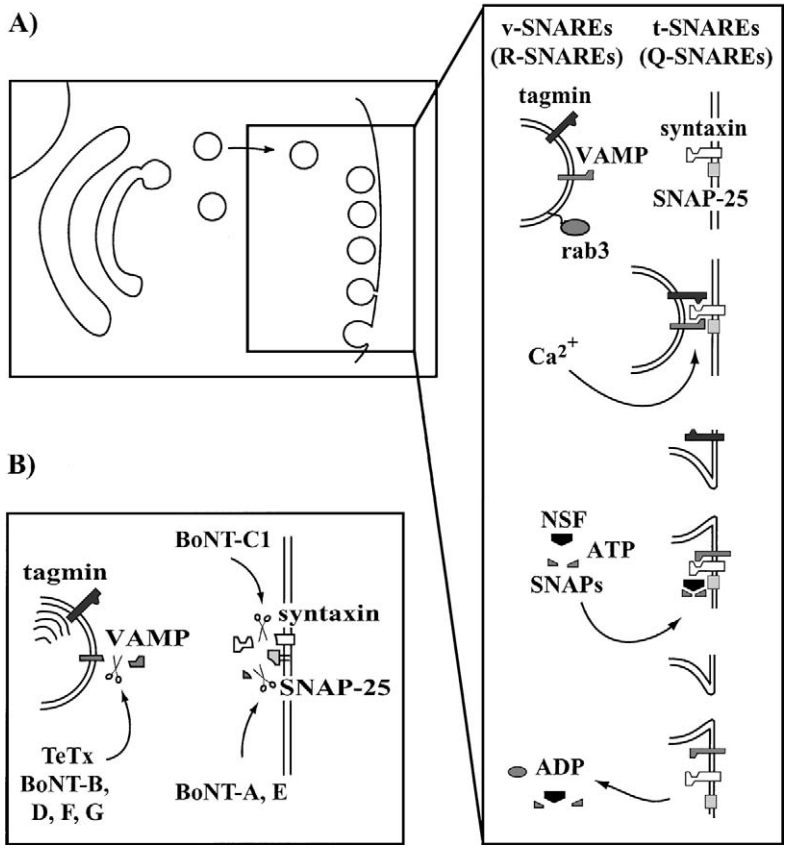



FIG. 15 Protein-mediated membrane fusion modeled with SNAREs. (A) Vesicle-associated v-SNAREs (VAMP and synaptotagmin) complex with cognate t-SNAREs (syntaxin and SNAP-25) to drive membrane fusion in response to $Ca^{+2}$ stimulus. The highly stable complex is then thought to be destabilized and broken apart by the action of soluble factors like NSF and SNAPs, recycling the SNARE proteins for another round of action. (B) Botulinum and tetanus neurotoxins can block vesicle fusion by specifically proteolyzing the various SNARE proteins at sequence specific sites.

*et al.,* 1998) and diverse members of these families are present throughout the secretory pathway of a cell. The X-ray crystal structure indicates that the SNARE heterotrimeric protein complex forms a four-helix bundle arranged in parallel (Hanson *et al.,* 1997; Poirier *et al.,* 1998b; Sutton *et al.,* 1998). It is the formation of this complex that is now believed to be a critical step leading to membrane fusion (Fasshauer *et al.,* 1998b; Jahn and Hanson, 1998; Weis and Scheller, 1998; Jahn, 2000; Jahn and Sudhof, 1999; Klenchin and Martin, 2000; Pinxteren *et al.,* 2000).

The clostridial neurotoxins Botulinum and tetanus (Fig. 15) functionally inhibit secretion by proteolytically cleaving members of the SNARE complex (Montecucco *et al.,* 1996). These neurotoxins are endopeptidases with exquisite specificity in a cell and have been used extensively in the functional analysis of SNARE-mediated membrane fusion. Specifically, tetanus and Botulinum neurotoxins B, D, F, and G cleaves VAMP/synaptobrevin (Montecucco and Schiavo, 1995), Botulinum C1 proteolyses syntaxin (Blasi *et al.,* 1993b), and Botulinum A and E cleave SNAP-25 (Blasi *et al.,* 1993a; Washbourne *et al.,* 1997). Synaptic vesicle fusion in neurons is prevented by treatment of cells with these sequence specific endopeptidases, likely by disrupting the ability of the individual SNARE components from complexing (Fasshauer *et al.,* 1998b). This hypothesis is due to the observation that the assembled complex is neurotoxin resistant, probably an effect of protection of the cleavage sites by tight complex conformation (Hayashi *et al.,* 1994; Poirier *et al.,* 1998a).

Membrane fusion is a calcium-stimulated event (see below) and following fusion, the SNARE complex is disassembled by action of the soluble ATPase, NSF (*n*-ethylmaleimide-sensitive fusion protein), and SNAPs (soluble NSF attachment proteins), enabling the recycling of subunits from an otherwise tightly complexed protein bundle (Fig. 15, Barnard *et al.,* 1997; Otto *et al.,* 1997). It is important to note that SNARE proteins and their associated soluble factors may not function universally in secretion. For example, secretion of peroxidase or cationic proteins in eosinophils (Lacy *et al.,* 1995) and apical secretion in MDCK cells (Ikonen *et al.,* 1995; see however, Low *et al.,* 1998) may function independent of fully formed SNARE complexes. However, recent advances in genome projects of many animals has resulted in the identification of dozens of family members for each of the SNARE proteins. In many cases, the overall sequence conservation of the family members is poor (less than 30%), yet the functional ability to participate in SNARE-mediated membrane fusion is retained. This complexity likely results in the subtle diversity and specialization of membrane fusion events throughout a cell.

In light of recent SNARE complex studies, an alternative classification scheme based on structurally important amino acids, rather than on preferred membrane localization use in the classic SNARE taxonomy, has been proposed. This change was proposed because the previous system did not hold for all vesicular transport events, such as homotypic fusion. Fasshauer and colleagues (Fasshauer *et al.,* 1998b) propose that SNARE proteins be reclassified into R- and Q-SNAREs. R-SNAREs contain a highly conserved arginine (R) residue which interacts with complementary glutamines of Q-SNAREs, an interaction important for SNARE complex stability. Thus, v- and t-SNAREs would be reclassified as Q- and R-SNAREs, respectively.

Recent evidence suggests that sea urchin eggs are functionally similar to the classic group of SNARE-dependent exocytic neurons. Steinhardt *et al.* (1994) examined membrane resealing in eggs following puncture as a function of vesicle

fusion (the vesicles were not identified) in sea urchin eggs and embryos. These authors concluded that membrane "healing" is an active process, induced by the influx of free calcium much like a triggered event of exocytosis. Membrane resealing events in eggs were blocked by Botulinum A and Botulinum B, which proteolyse SNAP-25 and cellubrevin/synaptobrevin/VAMP, respectively (Schiavo *et al.,* 1995a,b). This was the first published evidence suggesting that molecules of the SNARE hypothesis (Rothman and Wieland, 1996) are present in eggs.

These findings do not, however, demonstrate that such molecules are involved in cortical granule exocytosis and caution must be observed since an immunological screen for several molecules of the SNARE hypothesis yielded no cross-reacting proteins in the sea urchin egg (Whitaker, 1994). In addition, recall that somatic cells have been implicated to have mechanisms for regulated membrane fusion independent of the SNARE complex (Mellman, 1996).

Several lines of recent evidence, however, support a role for SNARE protein family members in mediating cortical granule fusion in the sea urchin egg. These include: (1) identification of the SNARE protein homologs of syntaxin and VAMP in the egg, which specifically associate with cortical granules and the plasma membrane (Fig. 16; Conner *et al.,* 1997; Conner and Wessel, 2001); (2) successful *in vitro* inhibition of fusion in sea urchin cortical lawns with tetanus toxin implicates VAMP function in cortical granule secretion (Avery *et al.,* 1997); and (3) cortical granule exocytosis is blocked when cortical granules were transiently undocked from the egg plasma membrane in the presence of Botulinum C1, which specifically proteolyzes syntaxin (Bi *et al.,* 1995). Thus, from these initial results we conclude that highly conserved SNARE protein family members are present in the sea urchin egg, particularly in association with cortical granules, and function as predicted to mediate the fusion of cortical granules with the plasma membrane at fertilization (Weber *et al.,* 1998).

## D. Calcium-Stimulated Membrane Fusion

Stimulus-dependent exocytosis is a calcium-dependent phenomenon in sea urchin eggs (Shen, 1995). While the SNARE complex can spontaneously form *in vitro* to drive membrane bilayer fusion in the absence of cytosolic factors (Weber *et al.,* 1998), membrane fusion is tightly controlled *in vivo* by proteins responsive to cytosolic calcium fluxes. What proteins regulate SNARE protein function and the calcium-dependent fusion of cortical granules with the plasma membrane at fertilization? It is likely that two types of proteins perform this task: (1) monomeric GTP-binding proteins of the rab family, which are required for vesicle transport and fusion, and (2) calcium-binding proteins which act as $Ca^{2+}$ sensors.

Rabs, monomeric GTPases belonging to the ras superfamily (rab, ras-like protein from rat brain) are essential for vesicle transport (reviewed by Schimmoller *et al.,* 1998; Woodman, 1998) and members of the rab3 family have been implicated to

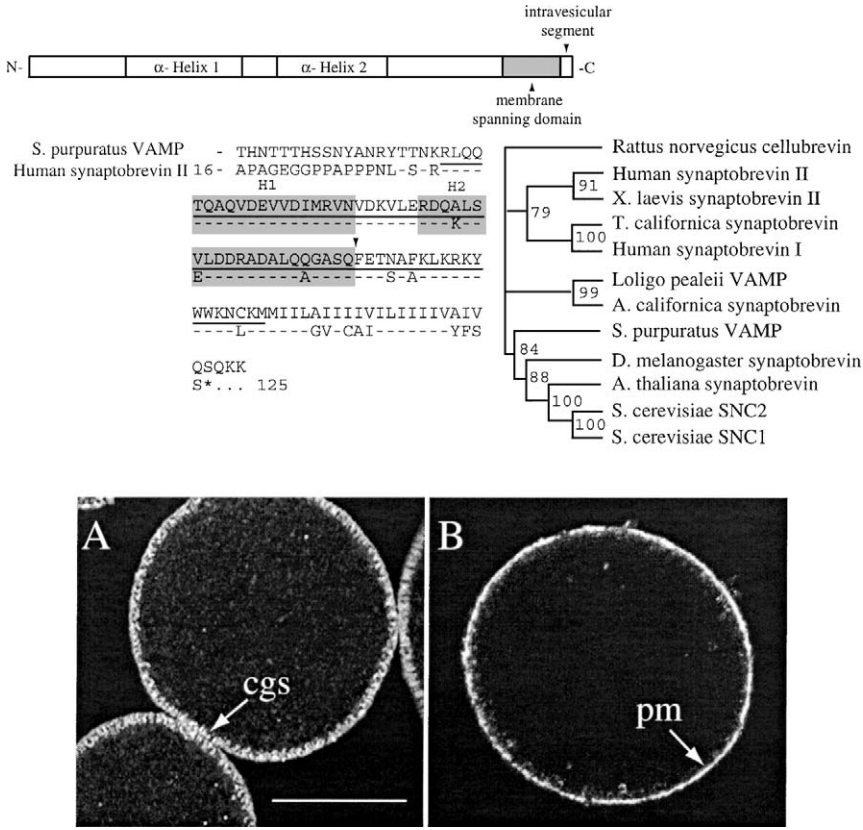



FIG. 16 Identification of SNARE homologs in sea urchin eggs. Shown is sea urchin VAMP, with its diagrammatic structure at top, followed by a sequence comparison with human VAMP (synaptobrevin II) sequence with the important helical domains involved in SNARE complex formation shaded, and the conserved site of tetanus toxin and *Botulinum* neurotoxin cleavage (arrow). At right is shown a cladogram of the VAMP family with bootstrap values inserted, and at bottom is the immunolocalization of VAMP in eggs either before or (A) or after fertilization (B). cgs, cortical granules; pm, plasma membrane. (Reproduced with permission from S. D. Connor, D. Leaf, and G. Wessel. Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Molecular Reproduction and Developments,* 1997;48:106–108.)

function in the final steps of the secretory pathway (Bean and Scheller, 1997). The first evidence that rabs function in the vesicular transport process came from the identification of a temperature-sensitive *Saccharomyces cerevisiae* mutant, sec4, that, when shifted to nonpermissive temperatures, accumulated secretory vesicles (Walworth *et al.,* 1989). Like SNARE proteins, different rab family members have been shown to localize to discrete secretory compartments, and most are ubiquitously expressed. Expression levels, however, vary from one cell type to another (reviewed by Martinez and Goud, 1998). Monomeric GTP-binding proteins are

defined by a nucleotide binding pocket formed by several highly conserved binding domains (reviewed by Sprang, 1997), cycling between active, GTP-bound, and inactive, GDP-bound states (Fig. 17; reviewed by Schimmoller *et al.,* 1998). In their active conformation, these proteins associate with membranes via a posttranslational covalent isoprenoid attachment at conserved C-terminal cysteine residues; mutations that prevent these modifications lead to functionally inactive proteins (Hancock *et al.,* 1989). GTP hydrolysis inactivates rabs and leads to a redistribution of the protein to the cytosol, the hydrophobic modification being shielded through cytosolic protein interactions (i.e., GDIs, rab cycle figure). G-protein activity is modulated by a variety of proteins which facilitate guanine nucleotide exchange and/or hydrolysis (Fig. 17). Rab activity is downregulated by GTPase activating proteins (GAPs), which enhance the low intrinsic GTPase activity and leave the protein in an inactive configuration. Rabs can then either be reactivated by exchanging GDP for GTP, requiring the action of guanine nucleotide exchange factors (GEFs), or repressed by guanine nucleotide dissociation inhibitors (GDIs, reviewed by Pfeffer *et al.,* 1995; Zerial and Stenmark, 1993). The exact function of these molecules and their role in vesicle docking and/or fusion has been difficult to elucidate since the secretory and the compensatory endocytic pathways are so dynamic (Bock and Scheller, 1997). Results from key experiments, however, have proposed several potential roles for rab: (1) regulating vesicle docking by controlling SNARE complex formation (Sogaard *et al.,* 1994); (2) modulating

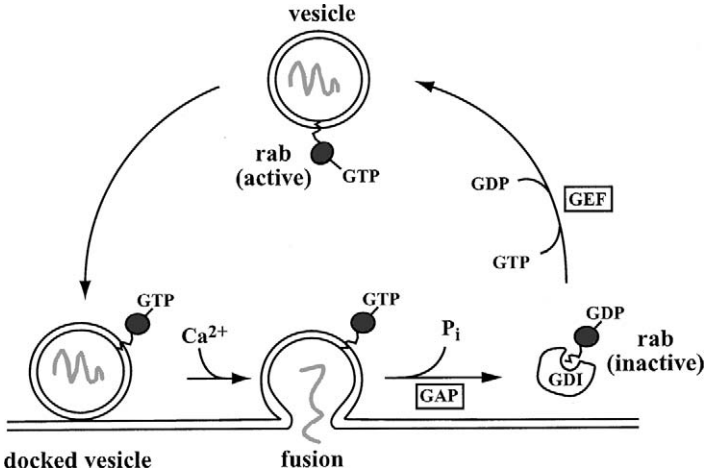
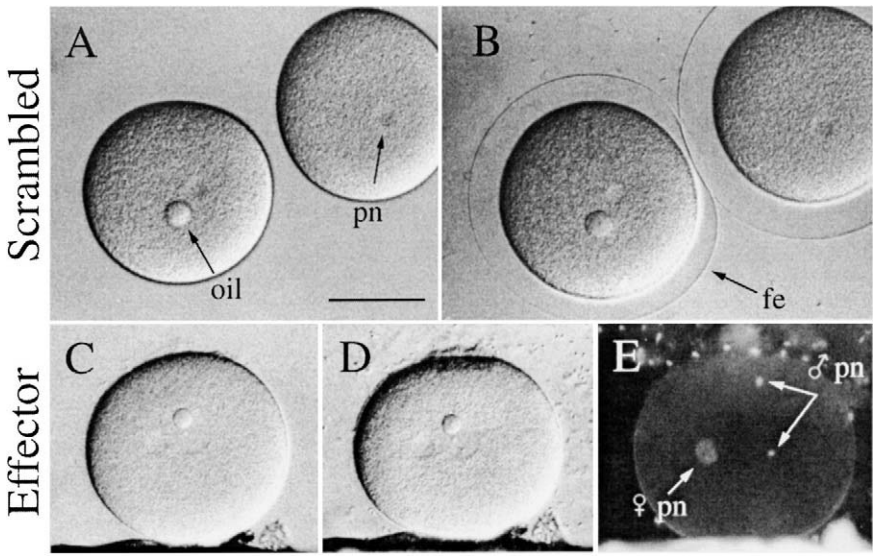



FIG. 17 The rab lifecycle. Rabs are recruited onto nascent vesicles in an active, GTP-bound state, anchored to the membrane via a C-terminal prenylation. Following vesicle fusion, the rab GTPase activity is enhanced by guanine nucleotide activating proteins (GAPs), resulting in GTP hydrolysis and a redistribution of rab protein into the cytosol through their association with guanine nucleotide dissociation inhibitors (GDIs). GDP/GTP exchange is then facilitated by guanine nucleotide exchange factors (GEFs), which reactivates rab proteins for another round of action.

SNARE complex stability (Johannes *et al.,* 1996); and/or (3) controlling the timing of vesicle fusion (Rybin *et al.,* 1996).

Rabs have been shown to transiently interact with a syntaxin to displace Sec1–Sly1 family members (also named *n*-Sec 1 or Munc 18), allowing the formation of the SNARE complex (reviewed by Lupashin and Waters, 1997; Schimmoller *et al.,* 1998; Sogaard *et al.,* 1994). Since thermolabile ypt 1 (a yeast rab family member) mutant yeast strains accumulate cytoplasmic vesicles, and protein analysis revealed a decreased in SNARE complex formation, it was concluded that rabs function in regulating vesicle docking at target membranes. It is feasible that cytoplasmic vesicle accumulation results from the saturation of either competent membrane surface area or vesicle docking machinery, while the lack of SNARE complex formation simply reflects a block in syntaxin interaction with other SNAREs which is normally facilitated by rabs. However, more recent results from rab3A-deficient mice suggest otherwise: while synaptic vesicle pool numbers and vesicle replenishing (docking) rates are normal in these rab3A$^{-/-}$ cells, calcium-stimulated exocytosis is altered, showing increased secretion only when neurotransmitter release is evoked (Geppert *et al.,* 1997). Thus, rab3A may be an initiator of vesicle fusion.

Recall that in the sea urchin, cortical granules are docked for prolonged periods of time (days to weeks) prior to exocytosis. Were rab3 functional only in docking of secretory granules to the plasma membrane, then cortical granule fusion should be independent of rab3 function. If, however, rab3 functions (also?) in the terminal fusion events, then we would expect inhibition of rab3 to block cortical granule secretion in eggs. A search for sea urchin rab3 was initiated and successfully resulted in a single homolog that associates selectively with cortical granules throughout oogenesis, translocation to the plasma membrane, docking, and up until cortical granule fusion (Conner *et al.,* 1997; Conner and Wessel, 1998). Following cortical granule exocytosis, rab3 relocalizes to distinct vesicle populations. To test for a function of rab3 following docking, function-blocking antibodies and peptides that block rab3 interaction with effector proteins were injected into eggs. This resulted in an effective block of cortical granule exocytosis and physiological polyspermy (Fig. 18). In addition, rab3 appears to function downstream of calcium signaling, since coinjection of the egg with the inhibitory peptide or antibody with IP3 fails to stimulate exocytosis. Thus, it was concluded that rab3 is essential for regulating cortical granule exocytosis *following* vesicle docking (Conner and Wessel, 1998). Combining these observations with the results that SNARE proteins function as the core vesicle fusion machinery (Weber *et al.,* 1998) and NSF functions following exocytosis to separate v- and t-SNAREs following membrane fusion (Nichols *et al.,* 1997; Ungermann *et al.,* 1998), we postulate a working model for the sea urchin egg: rab3 acts as a molecular switch on the complete formation of the SNARE complex, thereby deciding whether or not the complex can initiate the fusion of the cortical granule and the plasma membranes. When the egg is activated, the calcium wave will trigger an upstream effector of rab3 to stimulate




| Peptide | Concentration (μM) | n | % FE Formation | | |
|---|---|---|---|---|---|
| | | | Full | Partial | None |
| Water | 0 | 4 | 100 | 0 | 0 |
| Hypervariable | 218-315 | 8 | 100 | 0 | 0 |
| Scrambled effector | 218 | 18 | 100 | 0 | 0 |
| Effector | 24-48 | 14 | 93 | 0 | 7 |
| | 63-145 | 15 | 60 | 13 | 27 |
| | 218 | 37 | 11 | 49 | 40 |

FIG. 18 Cortical granule exocytosis, seen by fertilization envelope formation, is prevented by injection of the rab3 effecter peptide but not with the same amino acids scrambled in their sequence. pn, pronucleus; fe, fertilization envelope; n, number of eggs injected. (Reproduced with permission from S. D. Connor and G. Wessel. rab3 mediates cortical granule exocytosis in the sea urchin egg. *Developmental Biology,* 1998;203:334–344.)

completion of the partially assembled SNARE complex to allow for membrane fusion to occur.

This rab3 effector in sea urchins, however, has yet to be characterized. Recently, the calcium binding protein rabphilin3A has been shown to be important in calcium-mediated cortical granule fusion in the mouse oocyte (Masumoto *et al.,* 1996). This is an interesting observation since rabphilin3A has two calcium-binding C2 domains and has been shown to selectively interact with activated, GTP-bound rab3A (Shirataki *et al.,* 1993). The crystal structure of mammalian rab3A, complexed with the effector domain of rabphilin3A, has also been solved

(Ostermeier and Brunger, 1999). Structural analysis indicates that rabphilin3A directly contacts rab3A in two distinct regions: the first involves a region that overlaps the rab3 effector domain (the region used for peptides in rab3 functional analyses in the sea urchin egg) and is sensitive to the nucleotide binding state of rab3A; the second consists of a deep, hydrophobic pocket that may function in the intermolecular transduction of conformational changes. Such intimate contact at these critical sites makes it likely that rabphilin can regulate rab3 activity, which gives this calcium-binding protein a potentially important role in sea urchin cortical granule fusion.

Synaptotagmin, another calcium-binding protein associated with cortical granules has also been identified in the sea urchin. It, like VAMP and rab, is present selectively on cortical granules and redistributes to other membrane surfaces following exocytosis. Evidence that this protein is involved in the exocytosis of vesicles comes from many organisms: injection of competitive synaptotagmin calcium-binding-domain peptides into squid giant axons inhibits neurotransmitter release (Bommert *et al.,* 1993); *Drosophila* (DiAntonio and Schwarz, 1994), mouse, and *Caenorhabditis elegans* synaptotagmin knockouts show severely impaired neurotransmitter release (reviewed by Kelly, 1995; Schwarz, 1994); and a mutation in human synaptotagmin III has been associated with abnormal pancreatic secretion (Mizuta *et al.,* 1997). As with rabphilin3A, binding studies have shown that synaptotagmin cooperatively binds calcium through a conserved protein kinase C calcium-binding-motif (Davletov and Sudhof, 1993; Li *et al.,* 1995; Ubach *et al.,* 1998), and may associate with both SNAP-25 (Schiavo *et al.,* 1997) and syntaxin in a calcium-dependent manner (Chapman *et al.,* 1995; Kee and Scheller, 1996). Thus, synaptotagmin has been classified as a v-SNARE, making it a candidate liaison between the calcium wave at fertilization and cortical granule exocytosis. Direct proof of this interaction has yet to be determined, particularly since no proteolytic toxin against the synaptotagmin family of proteins is available.

If both rabs and calcium binding proteins play essential roles in cortical granule fusion, what is their connection? *In vitro,* calcium is the sole requirement for the fusion of isolated cortical granules attached to the plasma membrane (Crabb and Jackson, 1985; Vacquier, 1975b); therefore we conclude that calcium responsive protein(s) are upstream of the monomeric G-protein. Thus, it is possible that a calcium-binding protein, possibly rabphilin or synaptotagmin, in response to increases in intracellular calcium, either directly causes the activation of rab proteins, or initiates a cascade of protein interactions which stimulate rab function. If this model is correct, one should be able to either bypass the calcium requirement and induce vesicle fusion by artificial rab activation, or block G-protein function and inhibit calcium stimulated fusion. Consistent with this idea is the observation that rab3 competition with effector domain peptides blocks the calcium stimulated exocytosis of cortical granules in the sea urchin egg (Conner and Wessel, 1998). Though it is tempting to postulate that a single calcium-binding protein is involved

in the linear propagation of a signal resulting in vesicle fusion, the possibility that multiple calcium-dependent pathways are activated, necessitating different types of calcium binding proteins, cannot be ruled out.

## E. Conserved Mechanisms of Cortical Granule Exocytosis

Are the mechanisms that govern cortical granule exocytosis in the sea urchin conserved across phylogeny? While the details of cortical granule biology are less understood in other organisms (Ducibella *et al.,* 1994; Hoodbhoy and Talbot, 1994), several lines of evidence have implicated the functional role of SNARE and rab protein family members in mouse cortical granule exocytosis: (1) Injection of the neurotoxin Botulinum A, a SNAP-25-specific protease, significantly inhibits sperm-induced cortical granule exocytosis in mouse eggs, arguing that SNAP-25 is present and is functionally important for cortical granule fusion with the plasma membrane (Ikebuchi *et al.,* 1998). (2) Treatment of mouse eggs with N- or C-terminal peptides of rabphilin, a calcium-binding protein which interacts with rab family members, inhibits the cortical reaction in a concentration dependent manner in metaphase II mouse eggs (Masumoto *et al.,* 1996). (3) A rab3 family member is expressed in mouse eggs, and is enriched in the cortical region (Masumoto *et al.,* 1998). Given these observations, and the apparent functional conservation of SNARE and rab proteins in a broad range of cell types (Ferro-Novick and Jahn, 1994), it is likely that eggs from different phylogenetic groups share common mechanisms that mediate cortical granule fusion.

While SNARE and rab protein family members likely play key roles in the calcium-dependent membrane fusion of mouse and sea urchin cortical granules, it is unlikely that these proteins are the sole factors required. Cortical granule biology is unique in that vesicles can remain at the cortex for prolonged periods of time before being stimulated to fuse at fertilization. Mature, fertilizable eggs cannot tolerate sporadic vesicle fusion events like the "mini-spikes" observed in neurons, since the accumulated affects over time would result in a premature block to fertilization, a lethal consequence. Support for this idea comes from the observation that bovine chromaffin granules injected into immature Xenopus oocytes are capable of exocytosis in response to calcium stimulus while the endogenous cortical granules are refractory under similar stimulatory conditions (Scheuner and Holz, 1994). Chromaffin granules possess a battery of SNARE protein family members (Foran *et al.,* 1996; Hohne-Zell and Gratzl, 1996; Tagaya *et al.,* 1996; Tagaya *et al.,* 1995; Zimmermann, 1997) and likely employ the necessary *Xenopus* homologs to mediate vesicle fusion in the oocyte upon calcium stimulation. Thus, either *Xenopus* cortical granules do not employ SNAREs, or they possess unique factors that impose tight regulatory control over cortical granule exocytosis which may directly interact with those proteins demonstrated to have essential roles in other systems (i.e., SNAREs and rabs).

## F. Cortical Granule Fusion Competency

When are cortical granules competent for fusion with the egg plasma membrane? Cortical granules of the germinal vesicle (GV) stage sea urchin oocyte do not exocytose when challenged with sperm or the calcium ionophore A23187. This is not surprising, however, because cortical granules are docked in cells only following maturation (Berg and Wessel, 1997). In contrast to the sea urchin, treatment of GV-stage starfish oocytes with A23187 results in their exocytosis (Longo *et al.,* 1995). A similar responsiveness to ionophore or microinjection of stage IV frog oocytes with buffered $Ca^{2+}$ has been observed (Hollinger *et al.,* 1979; Hollinger and Schuetz, 1976), as well as activators of protein kinase C in prophase I oocytes (Bement and Capco, 1989). Treatment of GV-intact mouse oocytes with calcium ionophore does not induce the exocytosis of cortical granules. However, the ability of cortical granules to exocytose in response to A23187 increase following GVBD, reaching a maximum in the MII-arrested egg (Ducibella *et al.,* 1994). Similar results with IP3 injection have been reported (Ducibella *et al.,* 1993). Preovulatory mouse oocytes are incompetent to undergo the cortical reaction following induced calcium oscillations (Abbott *et al.,* 1999) even though it is well documented that roughly 30% of cortical granules spontaneously exocytose during meiotic maturation of the mouse oocyte (Ducibella *et al.,* 1995; Okada *et al.,* 1993) resulting in a cortical-free domain over metaphase II chromosomes of the mature oocyte (reviewed by Schultz and Kopf, 1995). This partial cortical granule release has been postulated to be required for the priming of the zona pellucida before the ZP reaction (Okada *et al.,* 1993); oddly though, not all mammalian eggs possess this cortical-free domain (Byers *et al.,* 1992). Based on these observations, it seems likely that cortical granules possess the appropriate machinery to target to the plasma membrane and the capacity to fuse before oocyte maturation. The ability of the oocyte to respond to appropriate translocation or fusion signals may, however, be limited by the signaling cascades responsible for transducing exocytosis (Ducibella, 1996).

## G. Membrane Recycling

In cells with active secretory roles, bursts of exocytosis are usually accompanied by a compensatory endocytic activity in an effort to maintain membrane surface area. This phenomenon has been documented in the nervous system of higher vertebrates, and is beautifully illustrated in cells of the frog neuromuscular junction (Heuser and Reese, 1981), the rat neurohypophysis (Nordmann and Artault, 1992), and the posterior pituitary gland (Nagasawa *et al.,* 1971).

In sea urchin eggs, cortical granules exocytosis at fertilization results in a greater than twofold increase in membrane surface area (Jaffe, 1996; Schroeder, 1979) and is rapidly followed by a resorption of membrane into large endocytic vesicles

~1.5 $\mu$m in diameter (Whalley *et al.,* 1995; Smith *et al.,* 2000). This phenomenon appears to be a general feature following the exocytosis of cortical granules in other organisms as well, including fish (Donovan and Hart, 1986), mammals (Kline and Stewart-Savage, 1994), and amphibians (Bernardini *et al.,* 1987; Bement *et al.,* 2000). While electron micrographs indicate that unfertilized sea urchin eggs possess clathrin plaques on the plasma membrane and following fertilization, coated pits rapidly form (Fisher and Rebhun, 1983; Sardet, 1984) and the bulk of membrane retrieval appears to be clathrin-independent (Whalley *et al.,* 1995) and requires extracellular $Ca^{2+}$ (Vogel *et al.,* 1999; Smith *et al.,* 2000). These observations are consistent with data obtained in adrenal chromaffin cells, where $Ca^{2+}$, GTP, and the GTPase dynamin are required for rapid exocytosis-coupled endocytosis while clathrin is not (Artalejo *et al.,* 1995).

## IX. Cortical Granules in Human Fertility Issues

Over 1% of natural human conceptions (Boue *et al.,* 1975) and approximately 10% of *in vitro* fertilizations (IVF) (Hammitt *et al.,* 1993) result in triploid embryonic abortions, the majority from polyspermic (mostly dispermic) fertilizations (Beatty, 1978; Jacobs *et al.,* 1978; Sakkas *et al.,* 1989). The increase in IVF polyspermic failures is likely the result of the use of much higher sperm concentrations than normally occur in the oviduct during natural conception. This is the normal practice, though, since fertilization failure is a common problem in clinical IVF, occurring in approximately 30% of all inseminated eggs. In addition, some patients present themselves as IVF infertile. Although the causes of fertilization failure are likely diverse and multiplicative, one candidate explanation for fertilization failure has recently been shown to involve cortical granules.

Ducibella and colleagues (Ducibella *et al.,* 1995) examined the cortical granules of unfertilized human eggs from failed IVF trials. These investigators noted a significant decrease in cortical granule number by use of the lectin (*L. culinaris* agglutinin, LCA) labeling assay. Since the authors could not compare the same oocytes before and after insemination, nor examine human eggs with known fertilization potential, they could not distinguish between the possibilities that these eggs originally had lower cortical granule numbers and the alternative, that cortical granules were spontaneously or prematurely exocytosed. To test the latter model, these investigators devised a biochemical technique of biotinylating the zona pellucida followed by gel electrophoresis and blotting, to monitor the mobility of the zona pellucida proteins. Shifts in the ZP2 to the smaller $ZP2_f$ would indicate whether or not the characteristic cortical granule-induced conversion in the zona pellucida had occurred. With this technique, they were able to monitor as little as 1/10 of the human zona pellucida proteins for biochemical conversion, and found that indeed, a significant mobility shift had occurred in the

zona proteins in half of their experimental samples, indicative of cortical granule exocytosis and processing of the zona proteins. Thus, fertilization failure in human cases appears to be frequently associated with spontaneous cortical granule exocytosis. The cause of the premature exocytosis is not known, but could occur within the patient prior to clinical retrieval, or be induced by the IVF conditions as a result of the ooctyes being extraordinarily susceptible to cytoplasmic activation, independent of the sperm.

Fertilization failure associated with cortical granule loss is also frequently observed in cryopreserved oocytes. Initially a high percentage of polyploidy was noted in oocytes previously cryopreserved both in mouse and human (Bouquet *et al.,* 1992; Eroglu *et al.,* 1998a,b; Laverge *et al.,* 1998). Although these investigators did not determine whether biochemical conversion in the zona pellucida had occurred as above, they did compare the ultrastructural morphology of frozen–thawed oocytes with normal (noncryopreserved) oocytes. Following processing for electron microscopic analysis, it was noted that the frozen–thawed oocytes consistently contained less cortical granules. Although it is difficult to diagnose based strictly on the ultrastructural morphology, it did appear as though the integrity of the cortical granules in the cryopreserved oocytes were compromised within the cytoplasm, and in some cases, cortical granule lysis was observed. In sibling oocytes used for IVF assays, a high incidence of polyploidy was noted, consistent with the conclusion that cortical granule exocytosis and the zona conversion was minimal, and ineffective at blocking additional sperm penetration. In separate experiments by other investigators, a loss of cortical granules was noted with a *reduced* rate of fertilization in mice, the opposite problem as above (Johnson *et al.,* 1988). This mouse phenotype is instead concluded to be the result of a premature zona reaction resulting from stimulated release of the cortical granules by the rapid cooling protocol, different from the above technique.

Thus, in addition to the basic biology offered by this interesting and beautiful organelle, cortical granules require important consideration when trying to maximize the efficacy of assisted reproductive technologies.

## X. Concluding Remarks

What began with Derbes in 1847 remains: a fascination with fertilization and a queue of new questions. What is clear now, two centuries removed, is that an integrated study of eggs and oocytes from diverse organisms is the most efficacious way of understanding this fundamental process of the life cycle. What the worm, the frog, the mouse, and the sea urchin offer are each different windows looking in at a highly efficient, smooth-running machine. We are currently assigning names to the gears and the levers, and with these pieces we need to be able to reassemble, with

our own understanding, a functional fertilization event. What is also abundantly clear, is that cortical granules offer a perspective on a fundamental process of regulated secretory granule biology that is distinct from the somatic cell window. Oocytes and their specialized vesicles have taken us full circle to the molecular level, as they did for Derbes at a phenomenological level in 1847.

## Acknowledgments

The PRovidence Institute of Molecular Oogenesis gratefully acknowledges the National Institutes of Health, the National Science Foundation, and the March of Dimes for their support of the work presented here.

## References

Abbott, A. L., Fissore, R. A., and Ducibella, T. (1999). Incompetence of preovulatory mouse oocytes to undergo cortical granule exocytosis following induced calcium oscillations. *Dev. Biol.* **207,** 38–48.

Adelson, D. L., Alliegro, M. C., and McClay, D. R. (1992). On the ultrastructure of hyalin, a cell adhesion protein of the sea urchin embryo extracellular matrix. *J. Cell Biol.* **116,** 1283–1289.

Adelson, D. L., and Humphreys, T. (1988). Sea urchin morphogenesis and cell-hyalin adhesion are perturbed by a monoclonal antibody specific for hyalin. *Development* **104,** 391–402.

Alliegro, M. C., Black, S. D., and McClay, D. R. (1992). Deployment of extracellular matrix proteins in sea urchin embryogenesis. *Microsc. Res. Tech.* **22,** 2–10.

Alliegro, M. C., Ettensohn, C. A., Burdsal, C. A., Erickson, H. P., and McClay, D. R. (1988). Echinonectin: A new embryonic substrate adhesion protein. *J. Cell Biol.* **107,** 2319–2327.

Alliegro, M. C., and Schuel, H. (1988). Immunocytochemical localization of the 35-kDa sea urchin egg trypsin-like protease and its effects upon the egg surface. *Dev. Biol.* **125,** 168–180.

Alves, A. P., Mulloy, B., Diniz, J. A., and Mourao, P. A. (1997). Sulfated polysaccharides from the egg jelly layer are species-specific inducers of acrosomal reaction in sperms of sea urchins. *J. Biol. Chem.* **272,** 6965–6971.

Alves, A. P., Mulloy, B., Moy, G. W., Vacquier, V. D., and Mourao, P. A. (1998). Females of the sea urchin Strongylocentrotus purpuratus differ in the structures of their egg jelly sulfated fucans. *Glycobiology* **8,** 939–946.

Anderson, E. (1968). Oocyte differentiation in the sea urchin, Arbacia punctulata, with particular reference to the origin of cortical granules and their participation in the cortical reaction. *J. Cell. Biol.* **37,** 514–539.

Anderson, E. (1974). Comparative aspects of the ultrastructure of the female gamete. *Int. Rev. Cytol.* **Suppl. 4,** 1–70.

Anstrom, J. A., Chin, J. E., Leaf, D. S., Parks, A. L., and Raff, R. A. (1988). Immunocytochemical evidence suggesting heterogeneity in the population of sea urchin egg cortical granules. *Dev. Biol.* **125,** 1–7.

Artalejo, C. R., Henley, J. R., McNiven, M. A., and Palfrey, H. C. (1995). Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca2+, GTP, and dynamin but not clathrin. *Proc. Natl. Acad. Sci. USA* **92,** 8328–8332.

Avery, J., Hodel, A., and Whitaker, M. (1997). In vitro exocytosis in sea urchin eggs requires a synaptobrevin-related protein. *J. Cell. Sci.* **110,** 1555–1561.

Babior, B. M. (1999). NADPH oxidase: An update. *Blood* **93,** 1464–1476.

Baca, M., and Zamboni, L. (1967). The fine structure of human follicular oocytes. *J. Ultrastruct. Res.* **19,** 354–381.

Bachman, E. S., and McClay, D. R. (1996). Molecular cloning of the first metazoan beta-1,3 glucanase from eggs of the sea urchin Strongylocentrotus purpuratus. *Proc. Natl. Acad. Sci. USA* **93,** 6808–6813.

Bajari, T. M., Lindstedt, K. A., Riepl, M., Mirsky, V. M., Nimpf, J., Wolfbeis, O. S., Dresel, H. A., Bautz, E. K., and Schneider, W. J. (1998). A minimal binding domain of the low density lipoprotein receptor family. *Biol. Chem.* **379,** 1053–1062.

Bakos, M. A., Kurosky, A., and Hedrick, J. L. (1990). Enzymatic and envelope-converting activities of pars recta oviductal fluid from Xenopus laevis. *Dev. Biol.* **138,** 169–176.

Bal, A. K. (1970). Selective staining of the ultra-structural components of cortical granules and golgi cisternae of sea urchin eggs. *Z. Zellforsch. Mikrosk. Anat.* **104,** 471–477.

Balinsky, B. I. (1966). Changes in the ultrastructure of amphibian eggs following fertilization. *Acta Embryol. Morphol. Exp.* **9,** 132–154.

Barkoff, A. F., Dickson, K. S., Gray, N. K., and Wickens, M. (2000). Translational control of cyclin B1 mRNA during meiotic maturation: Coordinated repression and cytoplasmic polyadenylation. *Dev. Biol.* **220,** 97–109.

Barnard, R. J., Morgan, A., and Burgoyne, R. D. (1997). Stimulation of NSF ATPase activity by alpha-SNAP is required for SNARE complex disassembly and exocytosis. *J. Cell Biol.* **139,** 875–883.

Bass, N. R., and Brafield, A. E. (1972). The life cycle of the polycheate Nereis virens. *J. Mar. Biol. Assoc. UK* **52,** 701–726.

Bean, A. J., and Scheller, R. H. (1997). Better late than never: A role for rabs late in exocytosis. *Neuron* **19,** 751–754.

Beatty, R. A. (1978). The origin of human triploidy: An integration of qualitative and quantitative evidence. *Ann. Hum. Genet.* **41,** 299–314.

Becker, K. A., and Hart, N. H. (1999). Reorganization of filamentous actin and myosin-II in zebrafish eggs correlates temporally and spatially with cortical granule exocytosis. *J. Cell. Sci.* **112,** 97–110.

Belton, R. J. Jr., and Foltz, K. R. (1995). Gamete and immune cell recognition revisited. *Bioessays* **17,** 1075–1080.

Bement, W., Benink, H., Mandato, C., and Sweltad, B. (2000). Evidence for direct membrane retieval following cortical granule exocytosis in *Xenopus* oocytes and eggs. *J. Exp. Zool.* **286,** 767–775.

Bement, W. M., and Capco, D. G. (1989). Intracellular signals trigger ultrastructural events characteristic of meiotic maturation in oocytes of Xenopus laevis. *Cell Tissue Res.* **255,** 183–191.

Bennett, M. K., and Scheller, R. H. (1993). The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA* **90,** 2559–2563.

Berg, L., and Wessel, G. (1997). Cortical granules of the sea urchin translocate early in oocyte maturation. *Dev. Suppl.* **124,** 1845–1850.

Berg, L. K., Chen, S. W., and Wessel, G. M. (1996). An extracellular matrix molecule that is selectively expressed during development is important for gastrulation in the sea urchin embryo. *Development* **122,** 703–713.

Bernardini, G., Ferraguti, M., and Stipani, R. (1987). Fertilization induces endocytosis in Xenopus eggs. *Cell Differ.* **21,** 255–260.

Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L., Gomez, J., De Baetselier, P., and Timmermans, M. (1998). Identification and cloning of a glucan- and lipopolysaccharide-binding protein from Eisenia foetida earthworm involved in the activation of prophenoloxidase cascade. *J. Biol. Chem.* **273,** 24,948–24,954.

Bi, G. Q., Alderton, J. M., and Steinhardt, R. A. (1995). Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol.* **131,** 1747–1758.

Bieri, S., Atkins, A. R., Lee, H. T., Winzor, D. J., Smith, R., and Kroon, P. A. (1998). Folding, calcium binding, and structural characterization of a concatemer of the first and second ligand-binding modules of the low-density lipoprotein receptor. *Biochemistry* **37,** 10,994–11,002.

Bieri, S., Djordjevic, J. T., Jamshidi, N., Smith, R., and Kroon, P. A. (1995). Expression and disulfide-bond connectivity of the second ligand-binding repeat of the human LDL receptor. *FEBS Lett.* **371,** 341–344.

Bisgrove, B. W., Andrews, M. E., and Raff, R. A. (1991). Fibropellins, products of an EGF repeat-containing gene, form a unique extracellular matrix structure that surrounds the sea urchin embryo. *Dev. Biol.* **146,** 89–99.

Bisgrove, B. W., and Raff, R. A. (1993). The SpEGF III gene encodes a member of the fibropellins: EGF repeat-containing proteins that form the apical lamina of the sea urchin embryo. *Dev. Biol.* **157,** 526–538.

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T. C., Niemann, H., and Jahn, R. (1993a). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* **365,** 160–163.

Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H., and Jahn, R. (1993b). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.* **12,** 4821–4828.

Bock, J. B., and Scheller, R. H. (1997). Protein transport. A fusion of new ideas. *Nature* **387,** 133–135.

Bommert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H., and Augustine, G. J. (1993). Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363,** 163–165.

Bonnell, B. S., and Hedrick, J. L. (1996). Purification and analysis of *Xenopus laevis* cortical granule lectin and its ligand. *Mol. Biol. Cell* **75,** 481a.

Bork, P., and Beckmann, G. (1993). The CUB domain. A widespread module in developmentally regulated proteins. *J. Mol. Biol.* **231,** 539–545.

Boue, J., Bou, A., and Lazar, P. (1975). Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions. *Teratology* **12,** 11–26.

Bouquet, M., Selva, J., and Auroux, M. (1992). The incidence of chromosomal abnormalities in frozen–thawed mouse oocytes after in-vitro fertilization. *Hum. Reprod.* **7,** 76–80.

Brown, M. S., and Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* **232,** 34–47.

Byers, A. P., Barone, M. A., Donoghue, A. M., and Wildt, D. E. (1992). Mature domestic cat oocyte does not express a cortical granule-free domain. *Biol. Reprod.* **47,** 709–715.

Carroll, E. J. Jr. (1976). Cortical granule proteases from sea urchin eggs. *Methods Enzymol.* **45,** 343–353.

Carroll, E. J., and Epel, D. (1975). Isolation and biological activity of the proteases released by sea urchin eggs following fertilization. *Dev. Biol.* **44,** 22–32.

Catterall, C. F., Lyons, A., Sim, R. B., Day, A. J., and Harris, T. J. (1987). Characterization of primary amino acid sequence of human complement control protein factor I from an analysis of cDNA clones. *Biochem. J.* **242,** 849–856.

Cecconi, S., Focarelli, R., Rossi, G., Talevi, R., and Colonna, R. (1998). Antral follicle development influences plasma membrane organization but not cortical granule distribution in mouse oocytes. *Hum. Reprod.* **13,** 2842–2847.

Chandler, D. E., and Heuser, J. (1979). Membrane fusion during secretion: Cortical granule exocytosis in sex urchin eggs as studied by quick-freezing and freeze-fracture. *J. Cell Biol.* **83,** 91–108.

Chandler, D. E., Whitaker, M., and Zimmerberg, J. (1989). High molecular weight polymers block cortical granule exocytosis in sea urchin eggs at the level of granule matrix disassembly. *J. Cell Biol.* **109,** 1269–1278.

Chapman, E. R., Hanson, P. I., An, S., and Jahn, R. (1995). Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. *J. Biol. Chem.* **270,** 23,667–23,671.

Chase, D. G. (1967). "Inhibition of the Cortical Reaction with High Hydrostatic Pressure and Its Effects on the Fertilization and Early Development of Sea Urchin Eggs.". Ph.D. thesis, University of Washington.

Chatlynne, L. G. (1969). A histochemical study of oogenesis in the sea urchin, Strongylocentrotus purpuratus. *Biol. Bull.* **136,** 167–184.

Chen, M. S., Tung, K. S., Coonrod, S. A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P. W., Herr, J. C., and White, J. M. (1999). Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6betal: Implications for murine fertilization. *Proc. Natl. Acad. Sci. USA* **96,** 11,830–11,835.

Cherr, G., Drobnis, E., and Katz, D. (1988). Localization of cortical granule constituents before and after exocytosis in the hamster egg. *J. Exp. Zool.* **246,** 81–93.

Chidgey, M. A. (1993). Protein targeting to dense-core secretory granules. *Bioessays* **15,** 317–321.

Citkowitz, E. (1971). The hyaline layer: its isolation and role in echinoderm development. *Dev. Biol.* **24,** 348–362.

Conner, S., Leaf, D., and Wessel, G. (1997). Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Mol. Reprod. Dev.* **48,** 106–118.

Conner, S., and Wessel, G. M. (1998). rab3 mediates cortical granule exocytosis in the sea urchin egg. *Dev. Biol.* **203,** 334–344.

Conner, S. D., and Wessel, G. M. (2001). Syntaxin, VAMP, and Rab3 are selectively expressed during sea urchin embryogensis. *Mol. Reprod. Dev.* **58,** 22–29.

Connors, S. A., Kanatsu-Shinohara, M., Schultz, R. M., and Kopf, G. S. (1998). Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. *Dev. Biol.* **200,** 103–115.

Cool, D. R., Fenger, M., Snell, C. R., and Loh, Y. P. (1995). Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway. *J. Biol. Chem.* **270,** 8723–8729.

Coorssen, J. R., Blank, P. S., Tahara, M., and Zimmerberg, J. (1998). Biochemical and functional studies of cortical vesicle fusion: The SNARE complex and Ca2+ sensitivity. *J. Cell Biol.* **143,** 1845–1857.

Crabb, J. H., and Jackson, R. C. (1985). In vitro reconstitution of exocytosis from plasma membrane and isolated secretory vesicles. *J. Cell Biol.* **101,** 2263–2273.

Cran, D. G., Moor, R. M., and Irvine, R. F. (1988). Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate. *J. Cell Sci.* **91,** 139–144.

Dabike, M., and Preller, A. (1999). Cytoarchitecture of Caudiverbera caudiverbera stage VI oocytes: A light and electron microscope study. *Anat. Embryol. (Berl.)* **199,** 489–497.

Daly, N. L., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995a). Three-dimensional structure of the second cysteine-rich repeat from the human low-density lipoprotein receptor. *Biochemistry* **34,** 14,474–14,481.

Daly, N. L., Scanlon, M. J., Djordjevic, J. T., Kroon, P. A., and Smith, R. (1995b). Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA* **92,** 6334–6338.

Davidson, E. H. (1986). "Gene Activity in Early Development." Academic Press San Diego,

Davletov, B. A., and Sudhof, T. C. (1993). A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. *J. Biol. Chem.* **268,** 26,386–26,390.

Decker, G. L., Valdizan, M. C., Wessel, G. M., and Lennarz, W. J. (1988). Developmental distribution of a cell surface glycoprotein in the sea urchin Strongylocentrotus purpuratus. *Dev. Biol.* **129,** 339–349.

Decker, S. J., and Kinsey, W. H. (1983). Characterization of cortical secretory vesicles from the sea urchin egg. *Dev. Biol.* **96,** 37–45.

Deits, T., Farrance, M., Kay, E. S., Medill, L., Turner, E. E., Weidman, P. J., and Shapiro, B. M. (1984). Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchin egg. *J. Biol. Chem.* **259,** 13,525–13,533.

Derbes, M. (1847). Observations sur le mecanisme et les phenomenes qui accompagnent la formaiton de l'embryon chezl'oursin comestible. *Ann. Sci. Nat. Zool.* **8,** 80–98.

Detering, N. K., Decker, G. L., Schmell, E. D., and Lennarz, W. J. (1977). Isolation and characterization of plasma membrane-associated cortical granules from sea urchin eggs. *J. Cell Biol.* **75,** 899–914.

DiAntonio, A., and Schwarz, T. L. (1994). The effect on synaptic physiology of synaptotagmin mutations in Drosophila. *Neuron* **12,** 909–920.

Donovan, M. J., and Hart, N. H. (1986). Cortical granule exocytosis is coupled with membrane retrieval in the egg of Brachydanio. *J. Exp. Zool.* **237,** 391–405.

Doren, S., Landsberger, N., Dwyer, N., Gold, L., Blanchette-Mackie, J., and Dean, J. (1999). Incorporation of mouse zona pellucida proteins into the envelope of Xenopus laevis oocytes. *Dev. Genes Evol.* **209,** 330–339.

Dostalova, Z., Calvete, J. J., Sanz, L., and Topfer-Petersen, E. (1995a). Boar spermadhesin AWN-1. Oligosaccharide and zona pellucida binding characteristics. *Eur. J. Biochem.* **230,** 329–336.

Dostalova, Z., Calvete, J. J., and Topfer-Petersen, E. (1995b). Interaction of non-aggregated boar AWN-1 and AQN-3 with phospholipid matrices. A model for coating of spermadhesins to the sperm surface. *Biol. Chem. Hoppe Seyler* **376,** 237–242.

Downey, J. C., and Lambert, C. C. (1994). Attachment of the ascidian sperm surface egg receptor N-acetylglucosaminidase to the cell membrane. *Mol. Reprod. Dev.* **38,** 453–458.

Drewett, J. G., and Garbers, D. L. (1994). The family of guanylyl cyclase receptors and their ligands. *Endocr. Rev.* **15,** 135–162.

Ducibella, T. (1996). The cortical reaction and development of activation competence in mammalian oocytes. *Hum. Reprod. Update* **2,** 29–42.

Ducibella, T., Anderson, E., Albertini, D. F., Aalberg, J., and Rangarajan, S. (1988a). Quantitative Studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. *Dev. Biol.* **130,** 184–197.

Ducibella, T., Dubey, A., Gross, V., Emmi, A., Penzias, A. S., Layman, L., and Reindollar, R. (1995). A zona biochemical change and spontaneous cortical granule loss in eggs that fail to fertilize in in vitro fertilization. *Fertil. Steril.* **64,** 1154–1161.

Ducibella, T., Duffy, P., and Buetow, J. (1994). Quantification and localization of cortical granules during oogenesis in the mouse. *Biol. Reprod.* **50,** 467–473.

Ducibella, T., Kurasawa, S., Duffy, P., Kopf, G. S., and Schultz, R. M. (1993). Regulation of the polyspermy block in the mouse egg: Maturation-dependent differences in cortical granule exocytosis and zona pellucida modifications induced by inositol 1,4,5-trisphosphate and an activator of protein kinase C. *Biol. Reprod.* **48,** 1251–1257.

Ducibella, T., Kurasawa, S., Rangarajan, S., Kopf, G. S., and Schultz, R. M. (1990). Precocious loss of cortical granules during mouse oocyte meiotic maturation and correlation with an egg-induced modification of the zona pellucida. *Dev. Biol.* **137,** 46–55.

Ducibella, T., Rangarajan, S., and Anderson, E. (1988b). The development of mouse oocyte cortical reaction competence is accompanied by major changes in cortical vesicles and not cortical granule depth. *Dev. Biol.* **130,** 789–792.

Epel, D., Patton, C., Wallace, R. W., and Cheung, W. Y. (1981). Calmodulin activates NAD kinase of sea urchin eggs: An early event of fertilization. *Cell* **23,** 543–549.

Epel, D., Weaver, A. M., and Mazia, D. (1970). Methods for revoval of the vitelline membrane of sea urchin eggs. I. Use of dithiothreitol (Cleland Reagent). *Exp. Cell Res.* **61,** 64–68.

Epel, D., Weaver, A. M., Muchmore, A. V., and Schimke, R. T. (1969). Beta-1,3-glucanase of sea urchin eggs: Release from particles at fertilization. *Science* **163,** 294–296.

Epifano, O., Liang, L. F., Familari, M., Moos, M. C. Jr., and Dean, J. (1995). Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* **121,** 1947–1956.

Eroglu, A., Toner, M., Leykin, L., and Toth, T. L. (1998a). Cytoskeleton and polyploidy after maturation and fertilization of cryopreserved germinal vesicle-stage mouse oocytes. *J. Assist. Reprod. Genet.* **15,** 447–454.

Eroglu, A., Toth, T. L., and Toner, M. (1998b). Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. *Fertil. Steril.* **69,** 944–957.

Fallon, J. F., and Austin, C. R. (1967). Fine structure of gametes of Nereis limbata (Annelida) before and after interaction. *J. Exp. Zool.* **166,** 225–241.

Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997). Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module [see comments]. *Nature* **388,** 691–693.

Fasshauer, D., Eliason, W. K., Brunger, A. T., and Jahn, R. (1998a). Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. *Biochemistry* **37,** 10,354–10,362.

Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998b). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl. Acad. Sci. USA* **95,** 15,781–15,786.

Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* **370,** 191–193.

Fink, R. D., and McClay, D. R. (1985). Three cell recognition changes accompany the ingression of sea urchin primary mesenchyme cells. *Dev. Biol.* **107,** 66–74.

Fisher, G. W., and Rebhun, L. I. (1983). Sea urchin egg cortical granule exocytosis is followed by a burst of membrane retrieval via uptake into coated vesicles. *Dev. Biol.* **99,** 456–472.

Fleming, W. N., and Saacke, R. G. (1972). Fine structure of the bovine oocyte from the mature graafian follicle. *J. Reprod. Fertil.* **29,** 203–213.

Florman, H. M., and Wassarman, P. M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* **41,** 313–324.

Fodor, E. J., Ako, H., and Walsh, K. A. (1975). Isolation of a protease from sea urchin eggs before and after fertilization. *Biochemistry* **14,** 4923–4937.

Foerder, C. A., and Shapiro, B. M. (1977). Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. *Proc. Natl. Acad. Sci. USA* **74,** 4214–4218.

Foltz, K. R. (1995). Sperm-binding proteins. *Int. Rev. Cytol.* **163,** 249–303.

Foltz, K. R., and Shilling, F. M. (1993). Receptor-mediated signal transduction and egg activation. *Zygote* **1,** 276–279.

Foran, P., Lawrence, G. W., Shone, C. C., Foster, K. A., and Dolly, J. O. (1996). Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. *Biochemistry* **35,** 2630–2636.

Frank-Vaillant, M., Jessus, C., Ozon, R., Maller, J. L., and Haccard, O. (1999). Two distinct mechanisms control the accumulation of cyclin B1 and Mos in Xenopus oocytes in response to progesterone. *Mol. Biol. Cell* **10,** 3279–3288.

Gache, C., Niman, H. L., and Vacquier, V. D. (1983). Monoclonal antibodies to the sea urchin egg vitelline layer inhibit fertilization by blocking sperm adhesion. *Exp. Cell Res.* **147,** 75–84.

Galeati, G., Modina, S., Lauria, A., and Mattioli, M. (1991). Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. *Mol. Reprod. Dev.* **29,** 40–46.

Galindo, B. E., Beltran, C., Cragoe, E. J. Jr., and Darszon, A. (2000). Participation of a K(+) channel modulated directly by cGMP in the speract-induced signaling cascade of strongylocentrotus purpuratus sea urchin sperm. *Dev. Biol.* **221,** 285–294.

Garbers, D. L. (1989). Molecular basis of fertilization. *Annu. Rev. Biochem.* **58,** 719–742.

Garbers, D. L. (1990). Guanylate cyclase receptor family. *Recent Prog. Horm. Res.* **46,** 85–96.

Geppert, M., Goda, Y., Stevens, C. F., and Sudhof, T. C. (1997). The small GTP-binding protein Rab3A regulates a late step in synaptic vesicle fusion. *Nature* **387,** 810–814.

Gerton, G. L., and Hedrick, J. L. (1986). The vitelline envelope to fertilization envelope conversion in eggs of Xenopus laevis. *Dev. Biol.* **116,** 1–7.

Ghiglione, C., Lhomond, G., Lepage, T., and Gache, C. (1994). Structure of the sea urchin hatching enzyme gene. *Eur. J. Biochem.* **219,** 845–854.

Glabe, C. G., and Vacquier, V. D. (1977). Isolation and characterization of the vitelline layer of sea urchin eggs. *J. Cell Biol.* **75,** 410–421.

Goldstein, J. L., Brown, M. S., Anderson, R. G., Russell, D. W., and Schneider, W. J. (1985). Receptormediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1,** 1–39.

Gould, M. C., and Stephano, J. L. (1991). Peptides from sperm acrosomal protein that initiate egg development. *Dev. Biol.* **146,** 509–518.

Grainger, J. L., von Brunn, A., and Winkler, M. M. (1986). Transient synthesis of a specific set of proteins during the rapid cleavage phase of sea urchin development. *Dev. Biol.* **114,** 403–415.

Grey, R. D., Wolf, D. P., and Hedrick, J. L. (1974). Formation and structure of the fertilization envelope in Xenopus laevis. *Dev. Biol.* **36,** 44–61.

Gross, S. D., Schwab, M. S., Taieb, F. E., Lewellyn, A. L., Qian, Y. W., and Maller, J. L. (2000a). The critical role of the MAP kinase pathway in meiosis II in Xenopus oocytes is mediated by p90(Rsk). *Curr. Biol.* **10,** 430–438.

Gross, V. S., Wessel, G. M., Florman, H. M., and Ducibella, T. (2000b). A monoclonal antibody that recognizes mammalian cortical granules and a 32-kilodalton protein in mouse eggs. *Biol. Reprod.* **63,** 575–581.

Gulyas, B. J. (1979). Cortical granules of mammalian eggs. *Int. Rev. Cytol.* **63,** 357–392.

Guraya, S. S. (1982). Recent progress in the structure, origin, composition, and function of cortical granules in animal egg. *Int. Rev. Cytol.* **78,** 257–360.

Hagstom, B. E. (1956). Studies on polyspermy in sea urchins. *Arkiv. For. Zool.* **10.**

Haley, S. A., and Wessel, G. M. (1999). The cortical granule serine protease CGSPI of the sea urchin, Strongylocentrotus purpuratus, is autocatalytic and contains a low-density lipoprotein receptor-like domain. *Dev. Biol.* **211,** 1–10.

Hall, H. G. (1978). Hardening of the sea urchin fertilization envelope by peroxidase-catalyzed phenolic coupling of tyrosines. *Cell* **15,** 343–355.

Hall, H. G., and Vacquier, V. D. (1982). The apical lamina of the sea urchin embryo: Major glycoproteins associated with the hyaline layer. *Dev. Biol.* **89,** 168–178.

Hammitt, D. G., Syrop, C. H., Walker, D. L., and Bennett, M. R. (1993). Conditions of oocyte storage and use of noninseminated as compared with inseminated, nonfertilized oocytes for the hemizona assay. *Fertil. Steril.* **60,** 131–136.

Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **57,** 1167–1177.

Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90,** 523–535.

Harvery, E. B. (1956). "The American Arbacia and Other Sea Urchins." Princeton University Press, Princeton, NJ

Harvey, E. B. (1911). The mechanism of membrane formation and other early changes in developing sea urchins' eggs as bearing on the problem of artificial parthenogenesis. *J. Exp. Zool.* **8,** 355–376.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: Action of clostridial neurotoxins on assmbly. *EMBO J.* **13,** 5021–5061.

Hedrick, J. L., and Katagiri, C. (1988). *Bufo japonicus japonicus* and *Xenopus laevis* egg jellies contain structurally related antigens and cortical granule lectin ligands. *J. Exp. Zool.* **245,** 78–85.

Henson, J. H., Beaulieu, S. M., Kaminer, B., and Begg, D. A. (1990). Differentiation of a calsequestrin-containing endoplasmic reticulum during sea urchin oogenesis. *Dev. Biol.* **142,** 255–269.

Heuser, J. E., and Reese, T. S. (1981). Structural changes after transmitter release at the frog neuromuscular junction. *J. Cell Biol.* **88,** 564–580.

Hirohashi, N., and Lennarz, W. J. (1998). The 350-kDa sea urchin egg receptor for sperm is localized in the vitelline layer. *Dev. Biol.* **204,** 305–315.

Hohne-Zell, B., and Gratzl, M. (1996). Adrenal chromaffin cells contain functionally different SNAP-25 monomers and SNAP-25/syntaxin heterodimers. *FEBS Lett.* **394,** 109–116.

Hollinger., T. G., Dumont, J. N., and Wallace, R. A. (1979). Calcium-induced dehiscence of cortical granules in *Xenopus laevis* oocytes. *J. Exp. Zool.* **210,** 107–115.

Hollinger, T. G., and Schutz, A. W. (1976). "Cleavage" and cortical granule breakdown in *Rana pipins* oocytes induced by direct microinjection of calcium. *J. Cell Biol.* **71,** 395–401.

Hoodbhoy, T., Carroll, E. J. Jr., and Talbot, P. (2000). Relationship between p62 and p56, two proteins of the mammalian cortical granule envelope, and hyalin, the major component of the echinoderm hyaline layer, in hamsters. *Biol. Reprod.* **62,** 979–987.

Hoodbhoy, T., and Talbot., P. (1994). Mammalian cortical granules: Contents, fate, and function. *Mol. Reprod. Dev.* **39,** 439–448.

Hope, J. (1965). The fine structure of the developing follice of the rhesus ovary. *J. Ultrastruct. Res.* **12,** 592–610.

Humphreys, W. J. (1967). The fine structure of cortical granules in eggs and gastrulae of Mytilus edulis. *J. Ultrastruct. Res.* **17,** 314–326.

Hurley, J. C. (1995). Endotoxemia: Methods of detection and clinical correlates. *Clin. Microbiol. Rev.* **8,** 268–292.

Hylander, B. L., and Summers, R. G. (1981). The effect of local anesthetics and ammonia on cortical granule-plasma membrane attachment in the sea urchin egg. *Dev. Biol.* **86,** 1–11.

Hylander, B. L., and Summers, R. G. (1982). An ultrastructural immunocytochemical localization of hyalin in the sea urchin egg. *Dev. Biol.* **93,** 368–380.

Ikebuchi, Y., Masumoto, N., Matsuoka, T., Yokoi, M., Tahara, T., Tasaka, K., Miyake, A., and Murata, Y. (1998). SNAP-25 is essential for cortical granule exocytosis in mouse eggs. *Am. J. Physiol.* **274,** C1496–C1500.

Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., and Simons, K. (1995). Diffrent requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* **81,** 571–80.

Innerarity, T. L., Friedlander, E. J., Rall, S. C. Jr., Weisgraber, K. H., and Mahley, R. W. (1983). The receptor-binding domain of human apolipoprotein E. Binding of apolipoprotein E fragments. *J. Biol. Chem.* **258,** 12,341–12,347.

Innerarity, T. L., and Mahley, R. W. (1978). Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry* **17,** 1440–1447.

lzadyar., F., Zeinstra, E., and Bevers, M. M. (1998). Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of in vitro matured bovine oocytes. *Mol. Reprod. Dev.* **51,** 339–345.

lzadyar, F., Zhao, J., Van Tol, H. T., Colenbrander, B., and Bevers, M. M. (1999). Messenger RNA expression and protein localization of growth hormone in bovine ovarian tissue and in cumulus oocyte complexes (COCs) during in vitro maturation. *Mol. Reprod. Dev.* **53,** 398–406.

Jackson, R. C., Ward, K. K., and Haggerty, J. G. (1985). Mild proteolytic digestion restores exocytotic activity to N-ethylmaleimide-inactivated cell surface complex from sea urchin eggs. *J. Cell Biol.* **101,** 6–11.

Jacobs, P. A., Angell, R. R., Buchanan, I. M., Hassold, T. J., Matsuyama, A. M., and Manuel, B. (1978). The origin of human triploids. *Ann. Hum. Genet.* **42,** 49–57.

Jaffe, L. A. (1996). Egg Membranes during Fertilization. *In* "Molecular Biology of Membrane Transport Disorders" (S. G. Schultz, Ed.), pp. 367–378. Plenum Press, New York.

Jahn, R. (2000). Secl/Munc18 proteins: Mediators of membrane fusion moving to center stage. *Neuron* **27,** 201–204.

Jahn, R., and Hanson, P. I. (1998). Membrane fusion. SNAREs line up in new environment. *Nature* **393,** 14–15.

Jahn, R., and Sudhof, T. C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem.* **68,** 863–911.

Johannes, L., Doussau, F., Clabecq, A., Henry, J. P., Darchen, F., and Poulain, B. (1996). Evidence for a functional link between Rab3 and the SNARE complex. *J. Cell Sci.* **109,** 2875–2884.

Johnson, M. H., Pickering, S. J., and George, M. A. (1998). The influence of cooling on the properties of the zona pellucida of the mouse oocyte. *Hum.Reprod.* **3,** 383–387.

Journet, A., and Tosi, M. (1986). Cloning and sequencing of full-length cDNA encoding the precursor of human complement component Clr. *Biochem. J.* **240,** 783–787.

Just, E. E. (1919). The fertilization reaction in *Echinarachnius parma* I. Cortical response of egg to insemination. *Biol. Bull.* **36,** 1–10.

Just, M. L., and Lennarz, W. J. (1997). Reexamination of the sequence of the sea urchin egg receptor for sperm: Implications with respect to its properties. *Dev. Biol.* **184,** 25–30.

Kamei, N., Swanson, W. J., and Glabe, C. G. (2000). A rapidly diverging EGF protein regulates species-specific signal transduction in early sea urchin development. *Dev. Biol.* **225,** 267–276.

Kane, R. E. (1970). Direct isolation of the hyaline layer protein released from the cortical granules of the sea urchin egg at fertilization. *J. Cell. Biol.* **45,** 615–622.

Kane, R. E. (1973). Hyalin release during normal sea urchin development and its replacement after removal at fertilization. *Exp. Cell Res.* **81,** 301–311.

Karp, G. C., and Solursh, M. (1974). Acid mucopolysaccharide metabolism, the cell surface, and primary mesenchyme cell activity in the sea urchin embryo. *Dev.Biol.* **41,** 110–123.

Katagiri, C., Maeda, R., Yamashika, C., Mita, K., Sargent, T. D., and Yasumasu, S. (1997). Molecular cloning of Xenopus hatching enzyme and its specific expression in hatching gland cells. *Int. J. Dev. Biol.* **41,** 19–25.

Kay, E. S., and Shapiro, B. M. (1987). Ovoperoxidase assembly into the sea urchin fertilization envelope and dityrosine crosslinking. *Dev. Biol.* **121,** 325–334.

Kee, Y., and Scheller, R. H. (1996). Localization of synaptotagmin-binding domains on syntaxin. *J. Neurosci.* **16,** 1975–1981.

Kelly, R. B. (1995). Neural transmission. Synaptotagmin is just a calcium sensor. *Curr. Biol.* **5,** 257–259.

Kemp, N. E., and Istock, N. L. (1967). Cortical changes in growing oocytes and in fertilized or pricked eggs of Rana pipiens. *J. Cell. Biol.* **34,** 111–122.

Kim, M. G., Chen, C., Lyu, M. S., Cho, E. G., Park, D., Kozak, C., and Schwartz, R. H. (1999). Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics* **49,** 420–428.

Kinsey, W. H. (1986). Purification and properties of the egg plasma membrane. *Methods Cell Biol.* **27,** 139–152.

Kitamoto, Y., Yuan, X., Wu, Q., McCourt, D. W., and Sadler, J. E. (1994). Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. *Proc. Natl. Acad. Sci. USA* **91,** 7588–7592.

Kitamura, Y., and Katagiri, C. (1998). Characterization of the hatching enzyme from embryos of an anuran amphibian, Rana pirica. *Biochim. Biophys. Acta* **1387,** 153–164.

Kitazume, S., Kitajima, K., Inoue, S., Haslam, S. M., Morris, H. R., Dell, A., Lennarz, W. J., and Inoue, Y. (1996). The occurrence of novel 9-O-sulfated N-glycolyneuraminic acid-capped alpha2→5-Oglycolyl-linked oligo/polyNeu5Gc chains in sea urchin egg cell surface glycoprotein. Identification of a new chain termination signal for polysialyltransferase. *J. Biol. Chem.* **271,** 6694–6701.

Klebanoff, S. J., Foerder, C. A., Eddy, E. M., and Shapiro, B. M. (1979). Metabolic similarities between fertilization and phagocytosis. Conservation of a peroxidatic mechanism. *J. Exp. Med.* **149,** 938–953.

Klenchin, V. A., and Martin, T. F. (2000). Priming in exocytosis: attaining fusion-competence after vesicle docking. *Biochimie* **82,** 399–407.

Kline, D., and Stewart-Savage, J. (1994). The timing of cortical granule fusion, content dispersal, and endocytosis during fertilization of the hamster egg: an electrophysiological and histochemical study. *Dev. Biol.* **162,** 277–287.

Kornfeld, S. (1987). Trafficking of lysosomal enzymes. *FASEB J.* **1,** 462–468.

Kowal, R. C., Herz, J., Weisgraber, K. H., Mahley, R. W., Brown, M. S., and Goldstein, J. L. (1990). Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265,** 10771–10779.

Krauskopf, C. (1968a). [Electron microscopic studies of the structure of the oocyte and the 2-cell stage in the rabbit. I. Oocyte]. *Z. Zelforsch. Mikrosk. Anat.* **92,** 275–295.

Krauskopf, C. (1968b). [Electron microscopic studies of the structure of the oocyte and the 2-cell stage in the rabbit. II. Blastomeres]. *Z. Zellforsch. Mikrosk. Anat.* **92,** 296–312.

Kubo, H., Kawano, T., Tsubuki, S., Kawashima, S., Katagiri, C., and Suzuki, A. (1997). A major glycoprotein of Xenopus egg vitelline envelope, gp41, is a frog homolog of mammalian ZP3. *Dev. Growth Differ.* **39,** 405–417.

Kume, S., Muto, A., Aruga, J., Nakagawa, T., Michikawa, T., Furuichi, T., Nakade, S., Okano, H., and Mikoshiba, K. (1993). The Xenopus IP3 receptor: Structure, function, and localization in oocytes and eggs. *Cell* **73,** 555–570.

Kuo, R. C., Baxter, G. T., Thompson, S. H., Stricker, S. A., Patton, C., Bonaventura, J., and Epel, D. (2000). NO is necessary and sufficient for egg activation at fertilization. *Nature* **406,** 633–636.

Lacy, p., Thompson., N., Tian., M., Solari, R., Hide, I., Newman, T., and Gomperts, B. (1995). A survey of GTP-binding proteins and other potential key regulators of exocytotic secretion in eosinophils. *J. Cell Sci.* **108,** 3547–3556.

LaFleur, G. J. Jr., Horiuchi, Y., and Wessel, G. M. (1998). Sea urchin ovoperoxidase: Oocyte-specific member of a heme-dependent peroxidase superfamily that functions in the block to polyspermy. *Mech. Dev.* **70,** 77–89.

Laidlaw, M., and Wessel, G. M. (1994). Cortical granule biogenesis is active throughout oogenesis in sea urchins. *Development* **120,** 1325–1333.

Lambert, C., Goudeau, H., Franchet, C., Lambert, G., and Goudeau, M. (1997). Ascidian eggs block polyspermy by two independent mechanism: One at the egg plasma membrane, the other involving the follicle cells. *Mol. Reprod. Dev.* **48,** 137–143.

Lambert, C. C. (1989). Ascidian eggs release glycosidase activity which aids in the block against polyspermy. *Development* **105,** 415–420.

Lambert, C. C., and Goode, C. A. (1992). Glycolipid linkage of a polyspermy blocking glycosidase to the ascidian egg surface. *Dev. Biol.* **154,** 95–100.

Larabell, C., and Chandler, D. E. (1991). Fertilization-induced changes in the vitelline envelope of echinoderm and amphibian eggs: Self-assembly of an extracellular matrix. *J. Electron Microsc. Tech.* **17,** 294–318.

Larabell, C. A., and Chandler, D. E. (1988a). Freeze-fracture analysis of structural reorganization during meiotic maturation in oocytes of Xenopus laevis. *Cell Tissue Res.* **251,** 129–136.

Larabell, C. A., and Chandler, D. E. (1988b). In vitro formation of the "S" layer, a unique component of the fertilization envelope in Xenopus laevis eggs. *Dev. Biol.* **130,** 356–364.

Laverge, H., Van der Elst, J., De Sutter, P., Verschraegen-Spae, M. R., De Paepe, A., and Dhont, M. (1998). Fluorescent in-situ hybridization on human embryos showing cleavage arrest after freezing and thawing. *Hum. Reprod.* **13,** 425–429.

Lebeche, D., and Kaminer, B. (1992). Characterization of a calsequestrin-like protein from sea-urchin eggs. *Biochem. J.* **287,** 741–747.

Lepage, T., and Gache, C. (1989). Purification and characterization of the sea urchin embryo hatching enzyme. *J. Biol. Chem.* **264,** 4787–4793.

Lepage, T., Sardet, C., and Gache, C. (1992). Spatial expression of the hatching enzyme gene in the sea urchin embryo. *Dev. Biol.* **150,** 23–32.

Li, C., Davletov, B. A., and Sudhof, T. C. (1995). Distinct Ca2+ and Sr2+ binding properties of synaptotagmins. Definition of candidate Ca2+ sensors for the fast and slow components of neurotransmitter release. *J. Biol. Chem.* **270,** 24,898–24,902.

Liang, L., Soyal, S. M., and Dean, J. (1997). FIGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development* **124,** 4939–4947.

Liang, L. F., Chamow, S. M., and Dean, J. (1990). Oocyte-specific expression of mouse Zp-2: Developmental regulation of the zona pellucida genes. *Mol. Cell Biol.* **10,** 1507–1515.

Liang, L. F., and Dean, J. (1993). Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes. *Dev. Biol.* **156,** 399–408.

Lillie, F. R. (1911). Studies of fertilization in Nereis. I The cortical changes in the egg. II. Partial fertilization. *J. Morphol.* **22,** 361–393.

Lillie, F. R. (1919). "Problems of Fertilization." University of Chicago Press Chicago.

Lindsay, L. L., and Hedrick, J. L. (1995). Isolation and characterization of ovochymase, a chymotrypsin-like protease released during Xenopous laevis egg activation. *Dev. Biol.* **167,** 513–516.

Lindsay, L. L., Yang, J. C., and Hedrick, J. L. (1999). Ovochymase, a Xenopus laevis egg extracellular

protease, is translated as part of an unusual polyprotease. *Proc. Natl. Acad. Sci. USA* **96,** 11,253–11,258.

Liu, C., Litscher, E. S., Mortillo, S., Sakai, Y., Kinloch, R. A., Stewart, C. L., and Wassarman, P. M. (1996). Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proc. Natl. Acad. Sci. USA* **93,** 5431–5436.

Longo, F. J., and Anderson, E. (1970). A cytological study of the relation of the cortical reaction to subsequent events of fertilization in urethane-treated eggs of the sea urchin *Arbacia punctulata*. *J. Cell Biol.* **47,** 646–665.

Longo, F. J. (1985). Fine structure of the mammalian egg cortex. *Am. J. Anat.* **174,** 303–315.

Longo, F. J. (1997). "Fertilization.". Chapman and Hall, London, New York.

Longo, F. J., Woerner, M., Chiba, K., and Hoshi, M. (1995). Cortical changes in starfish (Asterina pectinifera) oocytes during 1-methyladenine-induced maturation and fertilisation/activation. *Zygote* **3,** 225–239.

Low, S. H., Chapin, S. J., Wimmer, C., Whiteheart, S. W., Komuves, L. G., Mostov, K. E., and Weimbs, T. (1998). The SNARE machinery is involved in apical plasma membrane trafficking in MDCK cells. *J. Cell Biol.* **141,** 1503–1513.

Lupashin, V. V., and Waters, M. G. (1997). t-SNARE activation through transient interaction with a rab-like guanosine triphosphatase. *Science* **276,** 1255–1258.

Luttmer, S. J., and Longo, F. J. (1985). Ultrastructure and morphometric observations of cortical endoplasmic reticulum in Arbacia, Spisula and mouse eggs. *Dev. Growth Differ.* **27,** 349–359.

Mahley, R. W. (1988). Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* **240,** 622–30.

Martinez, O., and Goud, B. (1998). Rab proteins. *Biochim. Biophys. Acta* **1404,** 101–112.

Masuda, R., and Dan, J. (1977). Studies on the annual reproductive cycle of the sea urchin and the acid phosphatase of relict ova. *Biol. Bull.* **153,** 577–568.

Masumoto, N., Ikebuchi, Y., Tahara, M., Yokoi, T., Tasaka, K., Miyake, A., and Murata, Y. (1988). Expression of Rab3A in the cortical region in mouse metaphase II eggs. *J. Exp. Zool.* **280,** 91–96.

Masumoto, N., Sasaki, T., Tahara, M., Mammoto, A., Ikebuchi, Y., Tasaka, K., Tokunaga, M., Takai, Y., and Miyake, A. (1996). Involvement of Rabphilin-3A in cortical granule exocytosis in mouse eggs. *J. Cell Biol.* **135,** 1741–1747.

Matese, J. C., and McClay, D. R. (1998). Cortical granule exocytosis is triggered by different thresholds of calcium during fertilisation in sea urchin eggs. *Zygote* **6,** 55–64, 65a.

Mauk, R., Jaworski, D., Kamei, N., and Glabe, C. G. (1997). Identification of a 97-kDa heat shock protein from S. franciscanus ovaries with 94% amino acid identity to the S. purpuratus egg surface receptor for sperm. *Dev. Biol.* **184,** 31–37.

McClay, D. R., and Fink, R. D. (1982). Sea urchin hyalin: appearance and function in development. *Dev. Biol.* **92,** 285–293.

McNeil, P. L., Vogel, S. S., Miyake, K., and Terasaki, M. (2000). Patching plasma membrane disruptions with cytoplasmic membrane. *J. Cell Sci.* **113,** 1891–1902.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* **12,** 575–625.

Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000). Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature* **404,** 302–307.

Metz, E. C., and Palumbi, S. R. (1996). Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Mol. Biol. Evol.* **13,** 397–406.

Millar, S. E., Lader, E., Liang, L. F., and Dean, J. (1991). Oocyte-specific factors bind a conserved upstream sequence required for mouse zona pellucida promoter activity. *Mol. Cell Biol.* **11,** 6197–6204.

Miller, B. J., Georges-Labouesse, E., Primakoff, P., and Myles, D. G. (2000). Normal fertilization occurs with eggs lacking the integrin alpha6beta1 and is CD9-dependent. *J. Cell Biol.* **149,** 1289–1296.

Miller, D. J., Gong, X., Decker, G., and Shur, B. D. (1993). Egg cortical granule N-acetyl-glucosaminidase is required for the mouse zona block to polyspermy. *J.Cell Biol.* **123,** 1431–1440.

Miller, D. J., Macek, M. B., and Shur, B. D. (1992). Complementarity between sperm surface beta-1,4- galactosyltransferse and egg-coat ZP3 mediates sperm-egg binding. *Nature* **357,** 589–593.

Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., and Mekada, E. (2000). Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287,** 321–324.

Mizuta, M., Kurose, T., Miki, T., Shoji-Kasai, Y., Takahashi, M., Seino, S., and Matsukura, S. (1997). Localization and functional role of synaptotagmin III in insulin secretory vesicles in pancreatic beta-cells. *Diabetes* **46,** 2002–2006.

Molinete, M., Irminger, J. C., Tooze, S. A., and Halban, P. A. (2000). Trafficking/sorting and granule biogenesis in the beta-cell [In Process Citation]. *Semin. Cell Dev. Biol.* **11,** 243–251.

Moller, C. C., and Wassarman, P. M. (1989). Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. *Dev. Biol.* **132,** 103–112.

Montecucco, C., Papini, E., and Schiavo, G. (1996). Bacterial protein toxins and cell vesicle trafficking. *Experientia* **52,** 1026–1032.

Montecucco, C., and Schiavo, G. (1995). Structure and function of tetanus and botulinum neurotoxins. *Q. Rev. Biophys.* **28,** 423–472.

Moore, K. L., and Kinsey, W. H. (1994). Identification of an abl-related protein tyrosine kinase in the cortex of the sea urchin egg: Possible role at fertilization. *Dev. Biol.* **164,** 444–455.

Motomura, I. (1941). Materials of the fertilization membrane in the eggs of Echinoderms. *Sci. Rep. Tohoku Imp. Univ.* **4,** 345–363.

Mozingo, N. M., Somers, C. E., and Chandler, D. E. (1994). Ultrastructure of the proteoliaisin-ovoperoxidase complex and its spatial organization within the Strongylocentrotus purpuratus fertilization envelope. *J. Cell Sci.* **107,** 2769–2777.

Muchmore, A. V., Epel, D., Weaver, A. M., and Schimke, R. T. (1969). Purification and properties of an exo-beta-D-1,3-glucanase from sea urchin eggs. *Biochim. Biophys. Acta* **178,** 551–560.

Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S., and Iozzo, R. V. (1992). Primary structure of the human heparan sulfate protoeglycan from basement membrane (HSPG2/perlecan). A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J. Biol. Chem.* **267,** 8544–8557.

Murray, G., Reed, C., Marsden, M., Rise, M., Wang, D., and Burke, R. D. (2000). The alphaBbetaC integrin is expressed on the surface of the sea urchin egg and removed at fertilization [In Process Citation]. *Dev. Biol.* **227,** 633–647.

Muta, T., Seki, N., Takaki, Y., Hashimoto, R., Oda, T., Iwanaga, A., Tokunaga, F., Iwaki, D., and Iwanaga, S. (1996). Horseshoe crab factor G: A new heterodimeric serine protease zymogen sensitive to (1 → 3)-beta-D-glucan. *Adv. Exp. Med. Biol.* **389,** 79–85.

Nagasawa, J., Douglas, W. W., and Schulz, R. A. (1971). Micropinocytotic origin of coated and smooth microvesicles ("synaptic vesicles") in neurosecretory terminals of posterior pituitary glands demonstrated by incorporation of horseradish peroxidase. *Nature* **232,** 341–342.

Nelson, S. H., and McClay, D. R. (1988). Cell polarity in sea urchin embryos: reorientation of cells occurs quickly in aggregates. *Dev. Biol.* **127,** 235–247.

Nichols, B. J., Ungermann, C., Pelham, H. R., Wickner, W. T., and Haas, A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. *Nature* **387,** 199–202.

Nicosia, S. V., and Mikhail, G. (1975). Cumuli oophori in tissue culture: Hormone production, ultrastructure, and morphometry of early luteinization. *Fertil. Steril.* **26,** 427–448.

Nicosia, S. V., Wolf, D. P., and Inoue, M. (1977). Cortical granule distribution and cell surface characteristics in mouse eggs. *Dev. Biol.* **57,** 56–74.

Niman, H. L., Hough-Evans, B. R., Vacquier, V. D., Britten, R. J., Lerner, R. A., and Davidson, E. H. (1984). Proteins of the sea urchin egg vitelline layer. *Dev. Biol.* **102,** 390–401.

Nishigaki, T., and Darszon, A. (2000). Real-time measurements of the interactions between fluorescent speract and its sperm receptor. *Dev. Biol.* **233,** 17–26.

Nishihara, T., Wyrick, R. E., Working, P. K., Chen, Y. H., and Hedrick, J. L. (1986). Isolation and

characterization of a lectin from the cortical granules of Xenopus laevis eggs. *Biochemistry* **25,** 6013–6020.

Niwa, T., Matsukawa, Y., Senda, T., Nimura, Y., Hidaka, H., and Niki, I. (1998). Acetylcholine activates intracellular movement of insulin granules in pancreatic beta-cells via inositol trisphosphate-dependent [correction of triphosphate-dependent] mobilization of intracellular Ca2+ [published erratum appears in *Diabetes* 1999. Jan; **48**(1), 233]. *Diabetes* **47,** 1699–1706.

Nomura, K., Hoshino, K., and Suzuki, N. (1999). The primary and higher order structures of sea urchin ovoperoxidase as determined by cDNA cloning and predicted by homology modeling [published erratum appears in *Arch. Biochem. Biopshys.* 2000 Feb 15; **374**(2), 403]. *Arch. Biochem. Biophys.* **367,** 173–184.

Nomura, K., Shimizu, T., Kinoh, H., Sendai, Y., Inomata, M., and Suzuki, N. (1997). Sea urchin hatching enzyme (envelysin): cDNA cloning and deprivation of protein substrate specificity by autolytic degradation. *Biochemistry* **36,** 7225–7238.

Nomura, K., and Suzuki, N. (1995). Sea urchin ovoperoxidase: Solubilization and isolation from the fertilization envelope, some structural and functional properties, and degradation by hatching enzyme. *Arch. Biochem. Biophys.* **319,** 525–534.

Noonan, D. M., Fulle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., Yamada, Y., and Hassell, J. R. (1991). The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J. Biol. Chem.* **266,** 22,939–22,947.

Nordmann, J. J., and Artault, J. C. (1992). Membrane retrieval following exocytosis in isolated neurosecretory nerve endings. *Neuroscience* **49,** 201–207.

Ochiai, M., and Ashida, M. (2000). A pattern-recognition protein for beta-1,3-glucan. The binding domain and the cDNA cloning of beta-1,3-glucan recognition protein from the silkworm, Bombyx mori. *J. Biol. Chem.* **275,** 4995–5002.

Ohbayashi, H., Mantoku, T., Yamamoto, T., Nomura, K., and Suzuki, N. (1998). Primary structure of a 120 kDa protein associated with the fucose sulfate glycoconjugate constituting the acrosome reaction-inducing substance of the sea urchin, Hemicentrotus pulcherrimus. *Dev. Growth Differ.* **40,** 641–650.

Ohlendieck, K., and Lennarz, W. J. (1995). Role of the sea urchin egg receptor for sperm in gamete interactions. *Trends Biochem. Sci.* **20,** 29–33.

Ohlendieck, K., Partin, J. S., and Lennarz, W. J. (1994). The biologically active form of the sea urchin egg receptor for sperm is a disulfide-bonded homo-multimer. *J. Cell Biol.* **125,** 817–824.

Okada, A., Inomata, K., and Nagae, T. (1993). Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. *Anat. Rec.* **237,** 518–526.

Ostermeier, C., and Brunger, A. T. (1999). Structural basis of Rab effector specificity: Crystal structure of the small G protein Rab3A complexed with the effector domain of rabphilin-3A. *Cell* **96,** 363–374.

Otto, H., Hanson, P. I., and Jahn, R. (1997). Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **94,** 6197–6201.

Palumbi, S. R. (1999). All males are not created equal: Fertility differences depend on gamete recognition polymorphisms in sea urchins. *Proc. Natl. Acad. Sci. USA* **96,** 12,632–12,637.

Pasteels, J. J. (1965). [Electron microscope study of the cortical reaction. I. The cortical fertilization reaction in Paracentrotus and its chronology. II. The cortical reaction in the unfertilized egg of Sabellaria alveolata]. *J. Embryol. Exp. Morphol.* **13,** 327–339.

Patterson, L. (2001). "Cortical Granules of Polycheates." Ph.D. thesis, University of Newcastle.

Pfeffer, S. R., Dirac-Svejstrup, A. B., and Soldati, T. (1995). Rab GDP dissociation inhibitor: Putting rab GTPases in the right place. *J. Biol. Chem.* **270,** 17,057–17,059.

Pierce, K. E., Siebert, M. C., Kopf, G. S., Schultz, R. M., and Calarco, P. G. (1990). Characterization and localization of a mouse egg cortical granule antigen prior to and following fertilization or egg activation. *Dev. Biol.* **141,** 381–392.

Pinxteren, J. A., O'Sullivan, A. J., Larbi, K. Y., Tatham, P. E., and Gomperts, B. D. (2000). Thirty years of stimulus-secretion coupling: From Ca(2+) to GTP in the regulation of exocytosis. *Biochimie* **82,** 385–393.

Poirier, M. A., Hao, J. C., Malkus, P. N., Chan, C., Moore, M. F., King, D. S., and Bennett, M. K. (1998a). Protease resistance of synataxin. SNAP-25. VAMP complexes. Implications for assembly and structure. *J. Biol. Chem.* **273,** 11,370–11,377.

Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y. K., and Bennett, M. K. (1998b). The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Biol.* **5,** 765–769.

Prody, G. A., Greve, L. C., and Hedrick, J. L. (1985). Purification and characterization of an *N*-acetyl-beta-D-glucosaminidase from cortical granules of *Xenopus laevis* eggs. *J. Exp. Zool.* **235,** 335–340.

Punnonen, E. L., Wilke, T., von Figura, K., and Hille-Rehfeld, A. (1996). The oligomeric state of 46-kDa mannose 6-phosphate receptor does not change upon intracellular recycling and binding of ligands. *Eur. J. Biochem.* **237,** 809–818.

Quill, T. A., and Hedrick, J. L. (1996). The fertilization layer mediated block to polyspermy in Xenopus laevis: Isolation of the cortical granule lectin ligand. *Arch. Biochem. Biophys.* **333,** 326–332.

Rankin, T., and Dean, J. (2000). The zona pellucida: Using molecular genetics to study the mammalian egg coat [In Process Citation]. *Rev. Reprod.* **5,** 114–121.

Rankin, T., Familari, M., Lee, E., Ginsberg, A., Dwyer, N., Blanchette-Mackie, J., Drago, J., Westphal, H., and Dean, J. (1996). Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development* **122,** 2903–2910.

Raz, T., Ben-Yosef, D., and Shalgi, R. (1998a). Segregation of the pathways leading to cortical reaction and cell cycle activation in the rat egg. *Biol. Reprod.* **58,** 94–102.

Raz, T., Skutelsky, E., Amihai, D., Hammel, I., and Shalgi, R. (1998b). Mechanisms leading to cortical reaction in the mammalian egg. *Mol. Reprod. Dev.* **51,** 295–303.

Reimer, C. L., and Crawford, B. J. (1995). Identification and partial characterization of yolk and cortical granule proteins in eggs and embryos of the starfish, Pisaster ochraceus. *Dev. Biol.* **167,** 439–457.

Reynolds, S. D., Angerer, L. M., Palis, J., Nasir, A., and Angerer, R. C. (1992). Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to tolloid and BMP-1. *Development* **114,** 769–786.

Roe, J. L., and Lennarz, W. J. (1990). Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. *J. Biol. Chem.* **265,** 8704–8711.

Romao, M. J., Kolln, I., Dias, J. M., Carvalho, A. L., Romero, A., Varela, P. F., Sanz, L., Topfer-Petersen, E., and Calvete, J. J. (1997). Crystal structure of acidic seminal fluid protein (aSFP) at 1.9 A resolution: a bovine polypeptide of the spermadhesin family. *J. Mol. Biol.* **274,** 650–660.

Rothman, J. E., and Warren, G. (1994). Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* **4,** 220–233.

Rothman, J. E., and Wieland, F. T. (1996). Protein sorting by transport vesicles. *Science* **272,** 227–34.

Runft, L. L., Watras, J., and Jaffe, L. A. (1999). Calcium release at fertilization of Xenopus eggs requires type I IP(3) receptors, but not SH2 domain-mediated activation of PLCgamma or G(q)-mediated activation of PLCbeta. *Dev. Biol.* **214,** 399–411.

Runnstrom, J. (1966). The vitelline membrane and cortical particles in sea urchin eggs and their function in maturation and fertilization. *Adv. Morphol.* **5,** 221–325.

Russell, D. W., Brown, M. S., and Goldstein, J. L. (1989). Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J. Biol. Chem.* **264,** 21,682–21,688.

Ryabova, L. V., Betina, M. I., and Vassetzky, S. G. (1986). Influence of cytochalasin B on oocyte maturation in Xenopus laevis. *Cell Differ.* **19,** 89–96.

Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, C., Goody, R., and Zerial, M. (1996). GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. *Nature* **383,** 266–269.

Sakkas, D., Trounson, A. O., and Kola, I. (1989). In vivo cleavage rates and viability obtained for early cleavage mouse embryos in co-culture with oviduct cells. *Reprod. Fertil. Dev.* **1,** 127–136.

Santella, L., De Riso, L., Gragnaniello, G., and Kyozuka, K. (1999). Cortical granule translocation during maturation of starfish oocytes requires cytoskeletal rerrangement triggered by InsP3-mediated Ca2+ release. *Exp. Cell Res.* **248,** 567–574.

Sanz, L., Calvete, J. J., Jonakova, V., and Topfer-Petersen, E. (1992). Boar spermadhesins AQN-1 and AWN are sperm-associated acrosin inhibitor acceptor proteins. *FEBS Lett.* **300,** 63–66.

Sanz, L., Calvete, J. J., Mann, K., Gabius, H. J., and Topfer-Petersen, E. (1993). Isolation and biochemical characterization of heparin-binding proteins from boar seminal plasma: A dual role for spermadhesins in fertilization. *Mol. Reprod. Dev.* **35,** 37–43.

Sardet, C. (1984). The ultrastructure of the sea urchin egg cortex isolated before and after fertilization. *Dev. Biol.* **105,** 196–210.

Sato, K., Tokmakov, A. A., Iwasaki, T., and Fukami, Y. (2000). Tyrosine kinase-dependent activation of phospholipase cgamma is required for calcium transient in xenopus egg fertilization [In Process Citation]. *Dev. Biol.* **224,** 453–469.

Sawada, H., Miura, M., Yokosawa, H., and Ishii, S. (1984). Purification and characterization of trypsin-like enzyme from sea urchin eggs: Substrate specificity and physiological role. *Biochem. Biophys. Res. Commun.* **121,** 598–604.

Scheuner, D., and Holz, R. W. (1994). Evidence that the ability to respond to a calcium stimulus in exocytosis is determined by the secretory granule membrane: Comparison of exocytosis of injected bovine chromaffin granule membranes and endogenous cortical granules in Xenopus laevis oocytes. *Cell. Mol. Neurobiol.* **14,** 245–257.

Schiavo, G., Rossetto, O., Tonello, F., and Montecucco, C. (1995a). Intracellular targets and metalloprotease activity of tetanus and botulism neurotoxins. *Curr. Top. Microbiol. Immunol.* **195,** 257–274.

Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H., and Montecucco, C. (1995b). Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* **270,** 10,566–10,570.

Schiavo, G., Stenbeck, G., Rothman, J. E., and Sollner, T. H. (1997). Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin- treated synapses. *Proc. Natl. Acad. Sci. USA* **94,** 997–1001.

Schimmoller, F., Simon, I., and Pfeffer, S. R. (1998). Rab GTPases, directors of vesicle docking. *J. Biol. Chem.* **273,** 22,161–22,164.

Schmell, E. D., and Gulyas, B. J. (1980). Mammalian sperm-egg recognition and binding in vitro. I. Specificity of sperm interactions with live and fixed eggs in homologous and heterologous inseminations of hamster, mouse, and guinea pig oocytes. *Biol. Reprod.* **23,** 1075–1085.

Schroeder, T. E. (1979). Surface area change at fertilization: resorption of the mosaic membrane. *Dev. Biol.* **70,** 306–326.

Schuel, H. (1985). Secretory functions of egg cortical granules. *In* "In Biology of Fertilization" (M. C. B. and M. A., Eds.), Vol. 3, pp. 1–44. Academic Press, San Diego.

Schuel, H., Kelly, J. W., Berger, E. R., and Wilson, W. L. (1974). Sulfated acid mucopolysaccharides in the cortical granules of eggs. Effects of quaternary ammonium salts on fertilization. *Exp. Cell Res.* **88,** 24–30.

Schuel, H., Wilson, W. L., Chen, K., and Lorand, L. (1973). A trypsin-like proteinase localized in cortical granules isolated from unfertilized sea urchin eggs by zonal centrifugation. Role of the enzyme in fertilization. *Dev. Biol.* **34,** 175–186.

Schultz, R. M., and Kopf, G. S. (1995). Molecular basis of mammalian egg activation. *Curr. Top. Dev. Biol.* **30,** 21–62.

Schumaker, V. N., Phillips, M. L., and Chatterton, J. E. (1994). Apolipoprotein B and low-density lipoprotein structure: Implications for biosynthesis of triglyceride-rich lipoproteins. *Adv. Protein Chem.* **45,** 205–248.

Schwarz, T. L. (1994). Genetic analysis of neurotransmitter release at the synapse. *Curr. Opin. Neurobiol.* **4,** 633–639.

Seki, N., Muta, T., Oda, T., Iwaki, D., Kuma, K., Miyata, T., and Iwanaga, S. (1994). Horseshoe crab (1,3)-beta-D-glucan-sensitive coagulation factor G. A serine protease zymogen heterodimer with similarities to beta-glucan-binding proteins [published erratum appears in *J. Biol. Chem.* 1995 Jan 13; **270**(2), 986]. *J. Biol. Chem.* **269,** 1370–1374.

Selman, K., and Anderson, E. (1975). The formation and cytochemical characterization of cortical granules in ovarian oocytes of the golden hamster (Mesocricetus auratus). *J. Morphol.* **147,** 251–274.

Shapiro, B. M. (1991). The control of oxidant stress at fertilization. *Science* **252,** 533–536.

Shapiro, B. M., Somers, C., and Weidman, P. J. (1989). Extracellular remodeling during fertilization. *In* "Cell Biology of Fertilization" (H. Schatten and G. Schatten, Eds.), pp. 251–276. Academic Press, San Diego.

Shen, S. S. (1995). Mechanisms of calcium regulation in sea urchin eggs and their activities during fertilization. *Curr. Top. Dev. Biol.* **30,** 63–101.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993). Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell Biol.* **13,** 2061–2068.

Shyu, A. B., Raff, R. A., and Blumenthal, T. (1986). Expression of the vitellogenin gene in female and male sea urchin. *Proc. Natl. Acad. Sci. USA* **83,** 3865–3869.

Smith, R. M., Baibakov, B., Ikebuchi, Y., White, B. H., Lambert, N. A., Kaczmarek, L. K., and Vogel, S. S. (2000). Exocytotic insertion of calcium channels constrains compensatory endocytosis to sites of exocytosis. *J. Cell Biol.* **148,** 755–767.

Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E., and Sollner, T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. *Cell* **78,** 937–948.

Somers, C. E., Battaglia, D. E., and Shapiro, B. M. (1989). Localization and developmental fate of ovoperoxidase and proteoliaisin, two proteins involved in fertilization envelope assembly. *Dev. Biol.* **131,** 226–235.

Somers, C. E., and Shapiro, B. M. (1991). Functional domains of proteoliaisin, the adhesive protein that orchestrates fertilization envelope assembly. *J. Biol. Chem.* **266,** 16,870–16,875.

Sprang, S. R. (1997). G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.* **66,** 639–678.

Steinhardt, R. A., Bi, G., and Alderton, J. M. (1994). Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science* **263,** 390–393.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211,** 157–176.

Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* **395,** 347–353.

Szollosi, D. (1967). Development of cortical granules and the cortical reaction in rat and hamster eggs. *Anat. Rec.* **159,** 431–446.

Szollosi, D. (1978). On the role of gap junctions between follicle cells and oocyte in the mammalian ovarian follicle. *Res. Commun. Chem. Pathol. Pharmacol.* **10,** 3–4.

Tagaya, M., Genma, T., Yamamoto, A., Kozaki, S., and Mizushima, S. (1996). SNAP-25 is present on chromaffin granules and acts as a SNAP receptor. *FEBS Lett.* **394,** 83–86.

Tagaya, M., Toyonaga, S., Takahashi, M., Yamamoto, A., Fujiwara, T., Akagawa, K., Moriyama, Y., and Mizushima, S. (1995). Syntaxin 1 (HPC-1) is associated with chromaffin granules. *J. Biol. Chem.* **270,** 15,930–15,933.

Tahara, M., Coorssen, J. R., Timmers, K., Blank, P. S., Whalley, T., Scheller, R., and Zimmerberg, J. (1998). Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. *J. Biol. Chem.* **273,** 33,667–33,673.

Talbot, C. F., and Vacquier, V. D. (1982). The purification and characterization of an exo-beta (1 going to 3)-glucanohydrolase from sea urchin eggs. *J.Biol. Chem.* **257,** 742–746.

Terasaki, M. (1995). Visualization of exocytosis during sea urchin egg fertilization using confocal microscopy. *J. Cell Sci.* **108,** 2293–2300.

Terasaki, M., Henson, J., Begg, D., Kaminer, B., and Sardet, C. (1991). Characterization of sea urchin egg endoplasmic reticulum in cortical preparations. *Dev. Biol.* **148,** 398–401.

Terasaki, M., and Jaffe, L. A. (1991). Organization of the sea urchin egg endoplasmic reticulum and its reorganization at fertilization. *J. Cell Biol.* **114,** 929–940.

Thompson, J. D., Plewniak, F., Thierry, J., and Poch, O. (2000). DbClustal: Rapid and reliable global multiple alignments of protein sequences detected by database searches. *Nucleic Acids Res.* **28,** 2919–2926.

Tian, J., Gong, H., and Lennarz, W. J. (1999). Xenopus laevis sperm receptor gp69/64 glycoprotein is a homolog of the mammalian sperm receptor ZP2. *Proc. Natl. Acad. Sci. USA* **96,** 829–834.

Tian, J., Gong, H., Thomsen, G. H., and Lennarz, W. J. (1997a). Gamete interactions in Xenopus laevis: Identification of sperm binding glycoproteins in the egg vitelline envelope. *J. Cell Biol.* **136,** 1099–1108.

Tian, J., Gong, H., Thomsen, G. H., and Lennarz, W. J. (1997b). Xenopus laevis sperm–egg adhesion is regulated by modifications in the sperm receptor and the egg vitelline envelope. *Dev. Biol.* **187,** 143–153.

Tooze, S. A., and Stinchcombe, J. C. (1992). Biogenesis of secretory granules. *Semin. Cell Biol.* **3,** 357–366.

Topfer-Petersen, E., Romero, A., Varela, P. F., Ekhlasi-Hundrieser, M., Dostalova, Z., Sanz, L., and Calvete, J. J. (1998). Spermadhesins: A new protein family. Facts, hypotheses and perspectives. *Andrologia* **30,** 217–324.

Truschel, M. T., Chambers, S. A., Wang, C. Y., and McClay, D. R. (1987). Stage-specific expression of beta-1, 3-glucanase in sea urchin embryos and hybrids. *J. Exp. Zool.* **244,** 215–222.

Ubach, J., Zhang, X., Shao, X., Sudhof, T. C., and Rizo, J. (1998). Ca2+ binding to synaptotagmin: How many Ca2+ ions bind to the tip of a C2-domain?. *EMBO J.* **17,** 3921–3930.

Ungermann, C., Nichols, B. J., Pelham, H. R., and Wickner, W. (1998). A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol.* **140,** 61–69.

Urch, U. A., Nishihara, T., and Hedrick, J. L. (1979). The use of radioiodinated protein substrates for the assay of trypsin and the hatching enzyme from the amphibian Xenopus laevis. *Anal. Biochem.* **100,** 352–356.

Vacquier, V. D. (1975a). The appearance of beta-1,3-glucanase in hatching embryos of the sea urchin, Echinometra vanbrunti. *Exp. Cell Res.* **93,** 202–206.

Vacquier, V. D. (1975b). The isolation of intact cortical granules from sea urchin eggs: Calcium ions trigger granule discharge. *Dev. Biol.* **43,** 62–74.

Vacquier, V. D. (1976). Isolated cortical granules: A model system for studying membrane fusion and calcium-mediated exocytosis. *J. Supramol. Struct.* **5,** 27–35.

Vacquier, V. D., and Moy, G. W. (1977). Isolation of bindin: The protein responsible for adhesion of sperm to sea urchin eggs. *Proc. Natl. Acad. Sci. USA* **74,** 2456–2460.

Vacquier, V. D., and Moy, G. W. (1997). The fucose sulfate polymer of egg jelly binds to sperm REJ and is the inducer of the sea urchin sperm acrosome reaction. *Dev. Biol.* **192,** 125–135.

Vacquier, V. D., Tegner, M. J., and Epel, D. (1972). Protease activity establishes the block against polyspermy in sea urchin eggs. *Nature* **240,** 352–353.

Vacquier, V. D., Tegner, M. J., and Epel, D. (1973). Protease released from sea urchin eggs at fertilization alters the vitelline layer and aids in preventing polyspermy. *Exp. Cell Res.* **80,** 111–119.

Varela, P. F., Romero, A., Sanz, L., Romao, M. J., Topfer-Petersen, E., and Calvete, J. J. (1997). The 2.4 A resolution crystal structure of boar seminal plasma PSP-I/PSP-II: A zona pellucida-binding glycoprotein heterodimer of the spermadhesin family built by a CUB domain architecture. *J. Mol. Biol.* **274,** 635–649.

Vater, C. A., and Jackson, R. C. (1989). Purification and characterization of a cortical secretory vesicle membrane fraction. *Dev. Biol.* **135,** 111–123.

Verhey, C. A., and Moyer, F. H. (1967). Fine structural changes during sea urchin oogenesis. *J. Exp. Zool.* **164,** 95–132.

Vilela-Silva, A. C., Alves, A. P., Valente, A. P., Vacquier, V. D., and Mourao, P. A. (1999). Structure of the sulfated alpha-L-fucan from the egg jelly coat of the sea urchin Strongylocentrotus franciscanus: Patterns of preferential 2-O- and 4-O-sulfation determine sperm cell recognition. *Glycobiology* **9,** 927–933.

Vo, L. H., and Hedrick, J. L. (2000). Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC in *Xenopus laevis. Biol. Reprod.* **62,** 766–774.

Vogel, S. S., Delaney, K., and Zimmerberg, J. (1991). The sea urchin cortical reaction. A model system for studying the final steps of calcium-triggered vesicle fusion. *Ann. NY Acad. Sci.* **635,** 35–44.

Vogel, S. S., Smith, R. M., Baibakov, B., Ikebuchi, Y., and Lambert, N. A. (1999). Calcium influx is required for endocytotic membrane retrieval. *Proc. Natl. Acad. Sci. USA* **96,** 5019–5024.

Vu, T. K. H., Liu, R. W., Haaksma, C. J., Tomasek, J. J., and Howard, E. W. (1997). Identification and cloning of the membrane-associated serine protease, hepsin, from mouse preimplantation embryos. *J. Biol. Chem.* **272,** 31,315–13,320.

Wall, D. A., and Patel, S. (1989). Isolation of plasma membrane complexes from Xenopus oocytes. *J. Membr. Biol.* **107,** 189–201.

Wallace, R. A., and Selman, K. (1990). Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. *J. Electron Microsc. Tech.* **16,** 175–201.

Walworth, N. C., Goud, B., Kabcenell, A. K., and Novick, P. J. (1989). Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO J.* **8,** 1685–1693.

Wang, W., Hosoe, M., Li, R., and Shioya, Y. (1997). Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. *Dev. Growth Differ.* **39,** 607–615.

Warburg, O. (1908). Beobachtungen uber die Oxydationsprozesse im Seeigelei. *Z. Physiol. Chem.* **57,** 1–16.

Washbourne, P., Pellizzari, R., Baldini, G., Wilson, M. C., and Montecucco, C. (1997). Botulinum neurotoxin types A and E require the SNARE motif in SNAP-25 for proteolysis. *FEBS Lett.* **418,** 1–5.

Wassarman, P. M. (1999). Mammalian fertilization: Molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell* **96,** 175–183.

Wassarman, P. M., Bleil, J. D., Florman, H. M., Greve, J. M., Roller, R. J., Salzmann, G. S., and Samuels, F. G. (1985). The mouse egg's receptor for sperm: what is it and how does it work? *Cold Spring Harbor Symp. Quant. Biol.* **50,** 1–9.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998). SNAREpins: Minimal machinery for membrane fusion. *Cell* **92,** 759–772.

Weidman, P. J., Kay, E. S., and Shapiro, B. M. (1985). Assembly of the sea urchin fertilization membrane: Isolation of proteoliaisin, a calcium-dependent ovoperoxidase binding protein. *J. Cell Biol.* **100,** 938–946.

Weis, W. I., and Scheller, R. H. (1998). Membrane fusion. SNARE the rod, coil the complex. *Nature* **395,** 328–329.

Weisgraber, K. H. (1994). Apolipoprotein E: Structure–function relationships. *Adv. Protein Chem.* **45,** 249–302.

Weisgraber, K. H., Innerarity, T. L., Harder, K. J., Mahley, R. W., Milne, R. W., Marcel, Y. L., and Sparrow, J. T. (1983). The receptor-binding domain of human apolipoprotein E. Monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258,** 12,348–12,354.

Wessel, G. M. (1989). Cortical granule-specific components are present within oocytes and accessory cells during sea urchin oogenesis. *J. Histochem. Cytochem.* **37,** 1409–1420.

Wessel, G. M. (1995). A protein of the sea urchin cortical granules is targeted to the fertilization envelope and contains an LDL-receptor-like motif. *Dev. Biol.* **167,** 388–397.

Wessel, G. M., Berg, L., Adelson, D. L., Cannon, G., and McClay, D. R. (1998). A molecular analysis

of hyalin—A substrate for cell adhesion in the hyaline layer of the sea urchin embryo. *Dev. Biol.* **193,** 115–126.

Wessel, G. M., Conner, S., Laidlaw, M., Harrison, J., and LaFleur, G. J. Jr. (2000). SFE1, a constituent of the fertilization envelope in the sea urchin is made by oocytes and contains low-density lipoprotein-receptor-like repeats [In Process Citation]. *Biol. Reprod.* **63,** 1706–1712.

Wessel, G. M., Marchase, R. B., and McClay, D. R. (1984). Ontogeny of the basal lamina in the sea urchin embryo. *Dev. Biol.* **103,** 235–245.

Wessel, G. M., Truschel, M. R., Chambers, S. A., and McClay, D. R. (1987). A cortical granule-specific enzyme, B-1,3-glucanase, in sea urchin eggs. *Gamete Res.* **18,** 339–348.

Whalley, T., Terasaki, M., Cho, M. S., and Vogel, S. S. (1995). Direct membrane retrieval into large vesicles after exocytosis in sea urchin eggs. *J. Cell Biol.* **131,** 1183–1192.

Whalley, T., and Whitaker, M. (1988). Exocytosis reconstituted from the sea urchin egg is unaffected by calcium pretreatment of granules and plasma membrane. *Biosci. Rep.* **8,** 335–343.

Whitaker, M. (1994). Exocytosis in sea urchin eggs. *Ann. NY Acad. Sci.* **710,** 248–253.

Whitaker, M., and Swann, K. (1993). Lighting the fuse at fertilization. *Dev. Suppl.* **117,** 1–12.

Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991). Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science* **252,** 1817–1822.

Wolf, D. E., Kinsey, W., Lennarz, W., and Edidin, M. (1981). Changes in the organization of the sea urchin egg plasma membrane upon fertilization: indications from the lateral diffusion rates of lipid-soluble fluorescent dyes. *Dev. Biol.* **81,** 133–138.

Wolf, D. P. (1974a). The cortical granule reaction in living eggs of the toad, Xenopus laevis. *Dev. Biol.* **36,** 62–71.

Wolf, D. P. (1974b). On the contents of the cortical granules from Xenopus laevis eggs. *Dev. Biol.* **38,** 14–29.

Wolf, D. P., Nishihara, T., West, D. M., Wyrick, R. E., and Hedrick, J. L. (1976). Isolation, physico-chemical properties, and the macromolecular composition of the vitelline and fertilization envelopes from Xenopus laevis eggs. *Biochemistry* **15,** 3671–3678.

Woodman, P. (1998). Vesicle transport: more work for the Rabs?. *Curr. Biol.* **8,** R199–R201.

Wyrick, R. E., Nishihara, T., and Hedrick, J. L. (1974). Agglutination of jelly coat and cortical granule components and the block to polyspermy in the amphibian Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **71,** 2067–2071.

Yasumasu, S., Katow, S., Hamazaki, T. S., Iuchi, I., and Yamagami, K. (1992a). Two constituent proteases of a teleostean hatching enzyme: Concurrent syntheses and packaging in the same secretory granules in discrete arrangement. *Dev. Biol.* **149,** 349–356.

Yasumasu, S., Yamada, K., Akasaka, K., Mitsunaga, K., Iuchi, I., Shimada, H., and Yamagami, K. (1992b). Isolation of cDNAs for LCE and HCE, two constituent proteases of the hatching enzyme of Oryzias latipes, and concurrent expression of their mRNAs during development. *Dev. Biol.* **153,** 250–258.

Yazaki, I. (1968). Immunological analysis of the calcium precipitable protein of sea urchin eggs. (1) Hyaline layer substance. *Embryologia* **10,** 105–115.

Yurewicz, E. C., Oliphant, G., and Hedrick, J. L. (1975). The macromolecular composition of *Xenopus laevis* egg jelly coat. *Biochemistry* **14.**

Zerial, M., and Stenmark, H. (1993). Rab GTPases in vesicular transport. *Curr. Opin. Cell Biol.* **5,** 613–620.

Zhu, X., and Naz, R. K. (1999). Comparison of ZP3 protein sequences among vertebrate species: To obtain a consensus sequence for immunocontraception. *Front Biosci.* **4,** D212–D215.

Zimmermann, H. (1997). Are mechanisms of exocytosis neurotransmitter specific? *Neurochem. Int.* **31,** 759–761.

# Acetylcholinesterase Genes in the Nematode *Caenorhabditis elegans*

Didier Combes,[1] Yann Fedon,[1] Jean-Pierre Toutant,[1]
and Martine Arpagaus[1]
[1]Différenciation Cellulaire et Croissance/INRA, 34060 Montpellier Cedex 1, France

Acetylcholinesterase (AChE, EC 3.1.1.7) is responsible for the termination of cholinergic nerve transmission. It is the target of organophosphates and carbamates, two types of chemical pesticides being used extensively in agriculture and veterinary medicine against insects and nematodes. Whereas there is usually one single gene encoding AChE in insects, nematodes are one of the rare phyla where multiple *ace* genes have been unambiguously identified. We have taken advantage of the nematode *Caenorhabditis elegans* model to identify the four genes encoding AChE in this species. Two genes, *ace-1* and *ace-2,* encode two major AChEs with different pharmacological properties and tissue repartition: *ace-1* is expressed in muscle cells and a few neurons, whereas *ace-2* is mainly expressed in motoneurons. *ace-3* represents a minor proportion of the total AChE activity and is expressed only in a few cells, but it is able to sustain double null mutants *ace-1; ace-2*. It is resistant to usual cholinesterase inhibitors. *ace-4* was transcribed but the corresponding enzyme was not detected *in vivo*.

**KEY WORDS:** Acetylcholinesterases, Anticholinesterase pesticides,*Caenorhabditis elegans,* Cholinergic transmission, Nematodes, Pests.



## I. Introduction

At cholinergic synapses, the neurotransmitter ACh is synthesized by the enzyme choline acetyl transferase, released from the presynaptic side and hydrolyzed by a second enzyme, acetylcholinesterase (AChE, EC 3.1.1.7), after its interaction with

the postsynaptic ACh receptors (ACh-Rs). AChE is located within the synaptic cleft or is associated with plasma membranes of both pre- and postsynaptic cells. AChE fulfills an essential role in the transmission: when it is inhibited, increased amounts of ACh lead to the desensitization of ACh-Rs, the failure of transmission and ultimately to death. Common specific inhibitors of AChE are organophosphates and carbamates. These components are widely used in agriculture (chlorpyrifos, aldicarb) for fighting pests but are also active on beneficial insects. In addition, although less harmful to humans and other mammals because of body size and partial selectivity towards invertebrate AChEs, the margin of safety is often not great. It is thus necessary to have a complete knowledge of AChEs present in pest organisms that are treated by such chemical pesticides, and also in human, domestic animals and wild fauna, in order to improve as far as possible the selectivity and safety of pest treatments.

There is only one gene encoding AChE in vertebrates. Alternative splicing of two exons in the 3′ end of the coding region gives two peptides (H and T, respectively) differing in their C-termini. H peptides oligomerize into dimers anchored to the membrane through a glycolipid associated posttranslationally to the H subunit. T peptides can either tetramerize by interacting with an hydrophobic component that mediates the attachment to the membrane (amphiphilic G4 form of vertebrate brains) or be included in more complex molecular forms where catalytic subunits interact with a collagenic component ("asymmetric forms," up to molecular weight of 1000 kDa). Asymmetric forms have been considered to be the prominent functional forms at vertebrate neuromuscular junctions, whereas tetramers could be essential at central synapses. Recent reviews on vertebrate AChEs may be found in Massoulié *et al.* (1993) and Taylor and Radic (1994).

In most vertebrates, a second cholinesterase (butyrylcholinesterase, BChE, EC 3.1.1.8) is encoded by a distinct gene. This second cholinesterase is not primarily localized at cholinergic synapses. BChE does not exist in invertebrates and it likely appeared late during the evolution at the emergence of vertebrates (Toutant *et al.,* 1985; Sutherland *et al.,* 1997).

In invertebrates, excitatory innervation of neuromuscular junctions is either cholinergic (nematodes, annelids, mollusks) or glutaminergic (insects). So far, only one gene encoding acetylcholinesterase has been described in most species. In insects, for example, this single AChE gene encodes a glycolipid-anchored dimer of catalytic subunits which is found in cholinergic regions of the central nervous system (Toutant, 1989, for a review). An AChE gene encoding a similar molecular form has been recently found in the mollusc *Loligo* (Talesa *et al.,* 1999), where a very efficient cholinergic neuromuscular transmission takes place.

In the nematode *Caenorhabditis elegans,* three *ace* genes have been originally identified by genetics (Johnson *et al.,* 1988). A fourth gene has since been identified in *C. elegans* (Grauso *et al.,* 1998). We showed that expression of the multiple *ace* genes in nematodes results in a diversity of molecular forms of AChEs, expressed in different tissues. The molecular forms encoded by *ace* genes resemble, to some extent, globular forms found in vertebrates, but there is no asymmetric form.

## II. Cloning Four AChE Genes in *C. elegans*

The original work of C. D. Johnson, J. G. Culotti, and colleagues showed that two major classes of AChE coexisted in *C. elegans*. One class was Triton X-100-sensitive (class A) and the other was inhibited by deoxycholate (class B). Mutants, created by exposure to EMS, were then isolated that presented a reduced (or undetectable) amount of either class A or class B AChEs. This made it possible to localize *ace-1,* the structural gene encoding class A, on chromosome X, and *ace-2,* the gene encoding class B AChE, on chromosome I (Johnson *et al.,* 1981; Culotti *et al.,* 1981; Johnson and Russell, 1983). A novel class of AChE (class C) was defined later as the residual AChE activity detected in double mutants *ace-1; ace-2* (Kolson and Russell, 1985a,b). *ace-3,* the gene encoding class C, was mapped to chromosome II (Johnson *et al.,* 1988).

Our project to clone *ace* genes in *C. elegans* benefited of this earlier work and of several powerful advantages of this model nematode. First, we took advantage of the existence of a complete physical map of the genome as overlapping cosmid and yeast artificial chromosome clones (YACs) and of the public availability of YAC grids (YACs ordered by chromosomes, gift of J. Sulston and A. Coulson, Cambridge, UK). Second, we benefited from the public release of sequences from the genome sequencing projects for *C. elegans* and the related species *Caenorhabditis briggsae*. A complete description of these crucial points can be found in a recent review by Waterston *et al.* (1997).

### A. Cloning Strategy: Evidence for a Fourth *Ace* Gene

#### 1. *Ace-1*

In order to start cloning the *C. elegans ace* genes, we used PCR on genomic DNA with synthetic oligonucleotides derived from the two sequences 92-EDCLYLN-98 (sense primer) and 197-FGESAG-202 (reverse), which are highly conserved in the AChEs of vertebrates and of *Drosophila* (see the ESTHER database at http://www.ensam.inra.fr/cholinesterase and Cousin *et al.,* 1998b). A band of 700 bp was amplified on genomic DNA. This fragment was shown to hybridize to four YACs (Fig. 1) that precisely corresponded to the genetic location of *ace-1* (Arpagaus *et al.,* 1994). This gene was fully sequenced (including a large portion of the 5′ flanking region). Transformation of double mutants *ace-1; ace-2* (that display abnormal, uncoordinated locomotion) with the full gene was able to restore a coordinated mobility (Culetto *et al.,* 1999).

#### 2. *Ace-2*

Other PCR trials on genomic DNA or RNAs from N2 strains (wild-type) with the primers used to clone *ace-1* failed to amplify fragments other than *ace-1*.

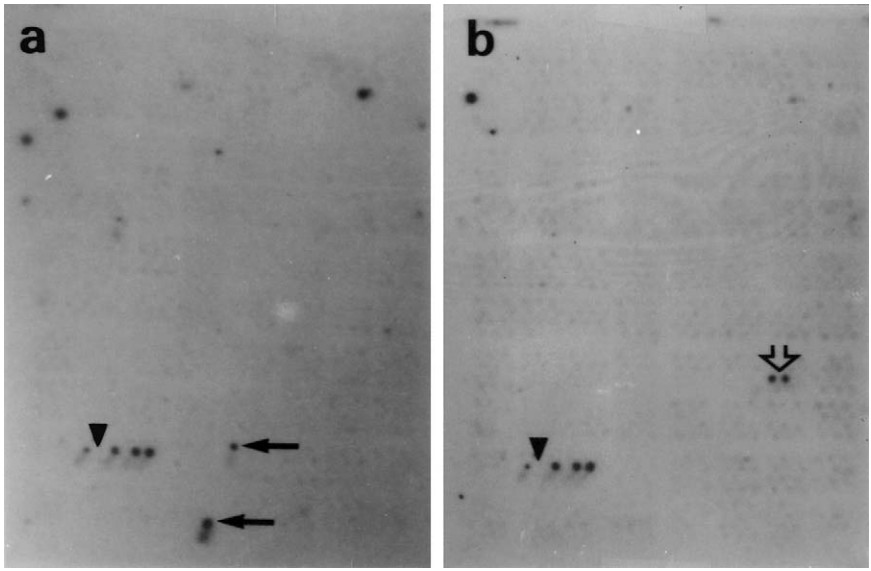



FIG. 1 Chromosome mapping of *ace* genes in *Caenorhabditis elegans.* The YAC polytene filter was hybridized with an *ace-1* probe (positive control of hybridization, arrowhead) in combination with either *ace-2* (part a, horizontal arrows) or with a PCR fragment of *ace-3* (part b, vertical arrow). In fact, *ace-3* and *ace-4* both hybridize to Y48B6 and Y59C8. Adapted from Grauso *et al.,* 1998, *FEBS Lett.* 424, 279–284, Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae.* Fig. 1, p. 280, with permission from Elsevier Science.

We then used total RNAs prepared from null mutants *ace-1*$^{-}$ (strain p1000) that possess a low amount of *ace-1* RNAs (Talesa *et al.,* 1995; see below). Sense oligonucleotides were designed from the peptides 80-GSEMWN-85 (*Torpedo* AChE) and 80-GTEMWN-85 (mammalian AChEs) and the reverse from the sequence 197-FGESAG-202. One band of 370 nt was amplified that proved to be a mixture of two sequences (40 and 48). Clone 40 hybridized to YACs Y44E3 and Y52G11 on chromosome I (Fig. 1a) and clone 48 to YACs Y48B6 and Y59C8 on chromosome II (Fig. 1b). We thus concluded that clones 40 and 48 were good candidates for *ace-2* and *ace-3,* respectively. We then obtained the full sequences (cDNA and genomic) corresponding to clone 40 in the two species *C. elegans* and *C. briggsae* and showed that it was able to restore a coordinated locomotion when injected into double mutants *ace-1; ace-2*. We thus concluded that we had cloned *ace-2*.

### 3. *Ace-3* and *Ace-4*

For further identification of clone 48, we searched a *C. elegans* genome database with this partial sequence, but the region had not been sequenced at that time. In contrast, we found two sequences in the *C. briggsae* BLAST server that presented homology with clone 48. These two sequences were contained within a single

phosmid (G18H21), indicating a close proximity of the two genes on chromosome II. We identified the two homologous genes in *C. elegans* and we obtained the full cDNA sequences by RT-PCR (both genes are transcribed). Genomic sequencing showed that there are only 356 nt between the stop codon of the upstream gene and the initiator ATG of the downstream gene (369 nt in *C. briggsae*). Thus both genes were at a location compatible with that of *ace-3* and it was therefore not possible to decide which one was in fact *ace-3* (defined as encoding class C AChE (Kolson and Russell, 1985a,b)). These two genes were provisionally called *ace-x* for the upstream gene and *ace-y* for the downstream gene (Grauso *et al.,* 1998). Sequencing of *ace-x* and *ace-y* in the null mutant *ace-3* (strain *ace-3*(p1304)II, Johnson *et al.,* 1988) showed finally a short deletion in *ace-y* but no mutation in *ace-x*. *ace-3* was therefore identified as the downstream gene of the tandem (*ace-y*) and the upstream gene (*ace-x*) was called *ace-4*.

## B. Chromosome Mapping of *ace* Genes

Using molecular probes specific for each *ace* gene, we refined their localization on the physical map of the genome. Figure 1 shows the hybridization of a YAC grid with such probes. The *ace-1* probe hybridized to YACs: Y45E8, Y49E3, Y50A2, and Y43C8. This indicates a position of *ace-1* near *let-2,* between *sdc-1* and *osm-1,* on the right arm of chromosome X (Arpagaus *et al.,* 1994). *ace-2* was found to hybridize to Y44E3 and Y52G11, two contiguous YACs of the left arm of chromosome 1. *ace-3* and *ace-4* both hybridized to the YACs Y48B6 and Y59C8 on the right arm of chromosome II (Fig. 1 and Grauso *et al.,* 1998).

## C. Characteristics of the Four Coding Sequences

The alignment of peptide sequences deduced from coding regions of *ace* cDNAs in *C. elegans* has been published recently (Combes *et al.,* 2000). It shows that the encoded proteins (ACE-1, ACE-2, ACE-3, and ACE-4) share the following characteristics of cholinesterases (by convention, numbering of amino acids in this paper is that of mature *Torpedo* AChE, not that of individual sequences; Bon *et al.,* 1986; Massoulié *et al.,* 1992). W84 is the choline binding site, and S200, E327, and H440 form a catalytic triad functioning as a charge relay system. Three pairs of cysteines, C67–C94, C254–C265, C402–C521, are involved into three internal disulfide bonds, and there are two potential salt bridges (R149–D172 and D397–R/K/H517). The sequence around the active serine (the characteristic FGES*AG of cholinesterases) was found to be modified in ACE-3 and ACE-4 (**V**GES*AG and FG**Q**S*AG, respectively, in both *C. elegans* and *C. briggsae*). In *Torpedo* AChE 14 aromatic residues line a substantial portion of the surface of the active gorge and could serve for "aromatic guidance" of the substrate down the gorge (Sussman

*et al.,* 1991). Of the 14 identified in *Torpedo* AChE, 12 are conserved or semiconserved in *ace-1,* 11 in *ace-2,* 12 in *ace-3,* and 12 in *ace-4* (see Table I). Finally it is noted that the C-terminal end of ACE-1 shows a remarkable homology with the C-terminus of T subunits of vertebrate AChEs, with an alternate disposition of charged and aromatic residues that could be organized as an amphiphilic $\alpha$ helix (see Fig. 2). In contrast to ACE-1, the C-terminal ends of ACE-2, ACE-3, and ACE-4 are clearly hydrophobic and possess a relatively polar stretch of amino acids where cleavage can take place during maturation, before addition of a glycolipid moiety. The peptidic region preceding the potential site of glycolipid addition contains a free cysteine in ACE-2, that could be involved in an interchain S–S bond. Such a free cysteine is absent from the C-terminal of ACE-3 and ACE-4 (Fig. 2).

Percentages of identity between amino acids sequences of the four ACEs are shown in Table II. ACE-1, ACE-2, and ACE-3 have relatively low identities (around 35%, similar to the identity of each ACE with *Drosophila* and *Torpedo* AChEs). In contrast, ACE-3 and ACE-4 are closer to each other (identity of 54%). They probably originate from a relatively recent duplication, before speciation of *C. elegans* and *C. briggsae,* which is estimated to be 40 Myr old (Kennedy *et al.,* 1993). Percentages of amino acid identity in homologous ACEs between *C. elegans* and *C. briggsae* were 94% (ACE-1), 91% (ACE-2), 90% (ACE-3), and 89% (ACE-4). Percentage of nucleotide identity between homologous *ace* genes in *C. elegans* and *C. briggsae* were 80% (*ace-1*), 81% (*ace-2*), 80% (*ace- 3*), and 81% (*ace-4*). In the four *ace* genes the active serine was encoded by TCn (see discussion in VII.A, below).

## D. Mutations

### 1. Phenotypes of Homozygous Null Mutants *ace-1, ace-2, ace-3,* and *ace-1; ace-2*

During the initial characterization of *ace-1, ace-2,* and *ace-3,* C. D. Johnson, J. G. Culotti, and colleagues reported that single null mutants in each of the three genes had no phenotype. In contrast the double null mutant, *ace-1; ace-2* was severely uncoordinated but viable (Culotti *et al.,* 1981; Johnson *et al.,* 1981, 1988). This observation led to the important conclusion that *ace-1* and *ace-2* were two major *ace* genes with overlapping functions (redundant genes that can compensate for each other). *ace-3* cannot compensate for either *ace-1* or *ace-2* but fulfills an important function, at least in *ace-1; ace-2* mutants, since the triple null mutant *ace-1; ace-2; ace-3* is lethal. This conclusion appears sound as far as gross phenotypes are concerned. However, extensive microscopic observation of *ace* mutants showed slight behavioral differences between single mutants in *ace* genes (Didier Combes, unpublished results).

TABLE I

Conservation of Aromatic Residues of the Active Gorge

| *Torpedo* AChE | Y 70 | W 84 | W 114 | Y 121 | Y 130 | W 233 | W 279 | F 288 | F 290 | F 330 | F 331 | Y 334 | W 432 | Y 442 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ACE-1 (12/14) | **S** | W | W | W | Y | W | W | **G** | F | Y | F | Y | W | Y |
| ACE-2 (11/14) | **T** | W | W | F | Y | W | **D** | **M** | F | Y | W | Y | W | Y |
| ACE-3 (12/14) | **G** | W | W | W | Y | W | W | **L** | F | F | W | Y | W | Y |
| ACE-4 (12/14) | **T** | W | W | Y | Y | W | W | **L** | F | Y | W | Y | W | Y |
| *Drosophila* AChE (10/14) | **E** | W | W | **M** | Y | W | W | **L** | F | Y | F | Y | F | **D** |
| Mammalian BChEs (8/14) | **N** | W | W | **Q** | Y | **W/V** | **A/R** | **L** | **V/I** | **A** | F | Y | F | Y |

*Note:* Fourteen aromatic residues lining the active gorge were identified in *Torpedo* AChE by Sussman *et al.* (1991). Corresponding residues are shown in *ace* genes (*C. elegans* and *C. briggsae*) as well as in *Drosophila* AChE and in mammalian BChEs. The number of conservation (same aromatic residue as in *Torpedo* sequence) and semiconservation (other aromatic residues than that in *Torpedo* sequence) at these 14 positions is indicated in parentheses.


FIG. 2 Comparison of C-terminals in nematode AChEs with known H and T subunits. C-terminal sequence encoded by exons 9 and 10 in ACE-1 of *Caenorhabditis* is aligned with the C-terminal end of AChE from *Meloidogyne incognita* and with the sequence encoded by exon T in *Torpedo californica* and *T. marmorata* AChEs. Arrows indicate the site of alternative splicing in *Torpedo* (i4) and the splicing sites of introns 8 and 9 in *Caenorhabditis ace-1*. Conserved aromatic residues are shown in bold. In *Nippostrongylus* AChE, the C-terminal end of the protein is located three residues downstream the splicing site common to all AChEs. In H subunits of *Torpedo,* rat, and *Drosophila* AChEs, the last residue of the mature protein ($\omega$-site) is shown in bold and the hydrophobic sequences which are cleaved and exchanged to GPIs are underlined. Note the series of three or four serines in C-termini of ACE-2, ACE-3, and ACE-4. The cleavage/GPI-attachment site of *Torpedo* AChE was found within such repeated serines (Bucht and Hjalmarson, 1996, Massoulié *et al.,* 1998). Using the prediction tool of Eisenbaher *et al.* (2000), available at http://mendel.imp.univie.ac.at/gpi/, $\omega$-sites were also found at those locations (open triangles). Cysteines residues implicated in interchain disulfide bonds are shown by vertical arrowheads. Such cysteines are present in ACE-1 and ACE-2, but not in ACE-3 nor ACE-4. (a) Schumacher *et al.,* 1986; (b) Sikorav *et al.,* 1988; (c) Arpagaus *et al.,* 1994, and Grauso *et al.,* 1996; (d) Piotte *et al.,* 1999; (e) Hussein *et al.,* 1999a; (f) Legay *et al.,* 1993; (g) Hall and Spierer, 1986; (h) Combes *et al.,* 2000; (i) Talesa *et al.,* 1999; (j) Bon *et al.,* 1988.

TABLE II
Percentages of Amino Acids Conservation (and Semiconservation) between AChEs

| | *ace-1* | *ace-2* | *ace-3* | *ace-4* | *Meloid.* | *Nippos.* | *Loligo* | *Droso.* | *Torpedo* |
|---|---|---|---|---|---|---|---|---|---|
| *ace-1* | | 35(52) | 34(53) | 36(55) | 64(80) | 30[a] | 46(62) | 35(50) | (T) 41(57) |
| *ace-2* | | | 36(53) | 35 (49) | 36(51) | 44(60) | — | 31(48) | (H) 35(51) |
| *ace-3* | | | | 54(71) | 34(55) | — | 32(50) | 36(56) | (H)34(54) |
| *ace-4* | | | | | 34(53) | 36(50) | 36(53) | 30(49) | (H) 37(53) |

*Note:* Data include the four *ace* genes of *Caenorhabditis elegans,* two other nematode AChEs: *Meloidogyne* (Piotte *et al.,* 1999) and *Nippostrongylus* (Hussein *et al.,* 1999a), the mollusc *Loligo* (Talesa *et al.,* 1999), and *Drosophila* (Hall and Spierer, 1986). *Torpedo* T and H sequences are from Schumacher *et al.* (1986) and Sikorav *et al.* (1988). Percentages of conservation (and of semiconservation) were obtained using BLASTP program. Full amino acids sequences of the four *ace* genes, including signal peptides and full C-termini, were searched successively. For comparison to *Torpedo* AChE, ACE-1 was compared to the T subunit while ACE-2, ACE-3, and ACE-4 were compared to the H subunit.

[a] This value is from Hussein *et al.* (1999a).

## 2. Molecular Basis of Null Mutations

We used the original strains of null mutants, created by exposure to EMS (Johnson *et al.,* 1981; Kolson and Russell, 1985a), provided either by C. D. Johnson, J. Rand (Oklahoma City, OK) or by the *Caenorhabditis* Genetics Center (St. Paul, MN). In the strain *ace-1*(p1000)X, we found an opal mutation that converts TGG (W*84*) into TGA (stop) (Talesa *et al.,* 1995). The corresponding non-sense messenger was found to be destabilized to only 10% of the amount of the normal *ace-1* mRNA (Talesa *et al.,* 1995). Sequencing of the full coding sequence of *ace-2* in the null mutant *ace2*(g72)I (a strain that lacks class B ACHE, Culotti *et al.,* 1981) showed also one point mutation G to A that changed G441 to E. The introduction of a negatively charged residue in the immediate proximity of H440 (a component of the charge relay) likely explains the loss of all ACE-2 activity in the mutant.

We sequenced both *ace-3* and *ace-4* genes in the mutant strain *ace-3*(p1304)II, which is a null *ace-3* mutant (no activity of class C AChE; see Johnson *et al.,* 1988). We found only a deletion of seven nucleotides after the codon AAG encoding K493 on both genomic DNA and cDNA of *ace-3*. The shift in reading frame introduces a stop codon TAG immediately downstream (Fig. 3B). Such a truncated coding sequence lacks the sixth cysteine involved in the third intrachain disulfide bond (Combes *et al.,* 2000). This likely prevents correct folding of the protein and thus a normal catalytic activity.

The mutant strain dc2 was also originally characterized as an *ace-3* mutant (*ace-3*(dc2)II; Johnson *et al.,* 1988). Sequencing genomic DNA in dc2 showed that a 581-nt-long deletion removed the 3′ end of the coding sequence of *ace-4* (50 nt), the whole intergenic region (356 nt), and the 5′ end of *ace-3* (175 nt)

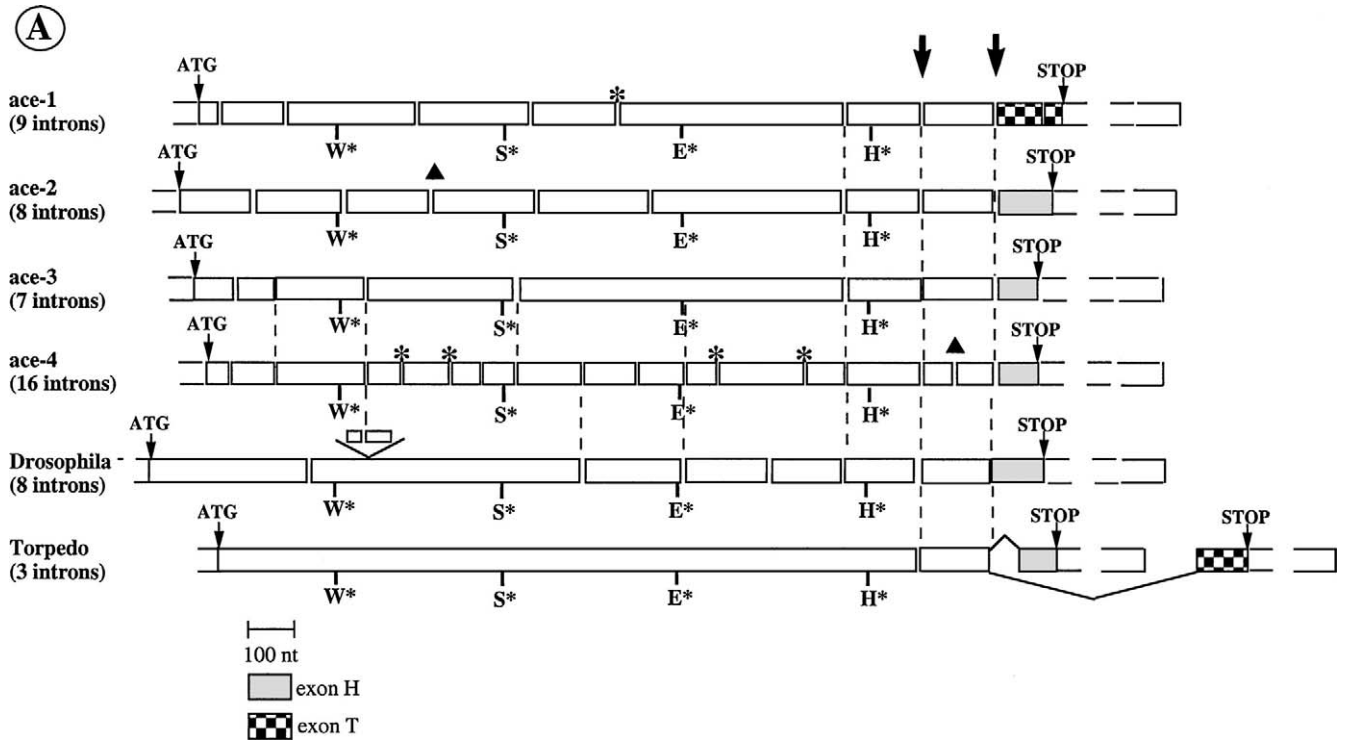
(see Fig. 3B). A single large transcript *ace-3–ace-4* was found in the mutant but no individual *ace-3* or *ace-4* mRNAs (Combes *et al.,* 2000). This large transcript could be translated into a single protein since the 581-nt-long deletion does not shift the reading frame. Misfolding of this large protein, however, likely explains the absence of class C activity and might as well prevent catalytic activity of ACE-4. dc2 mutant could thus be a double null mutant *ace-3; ace-4*.

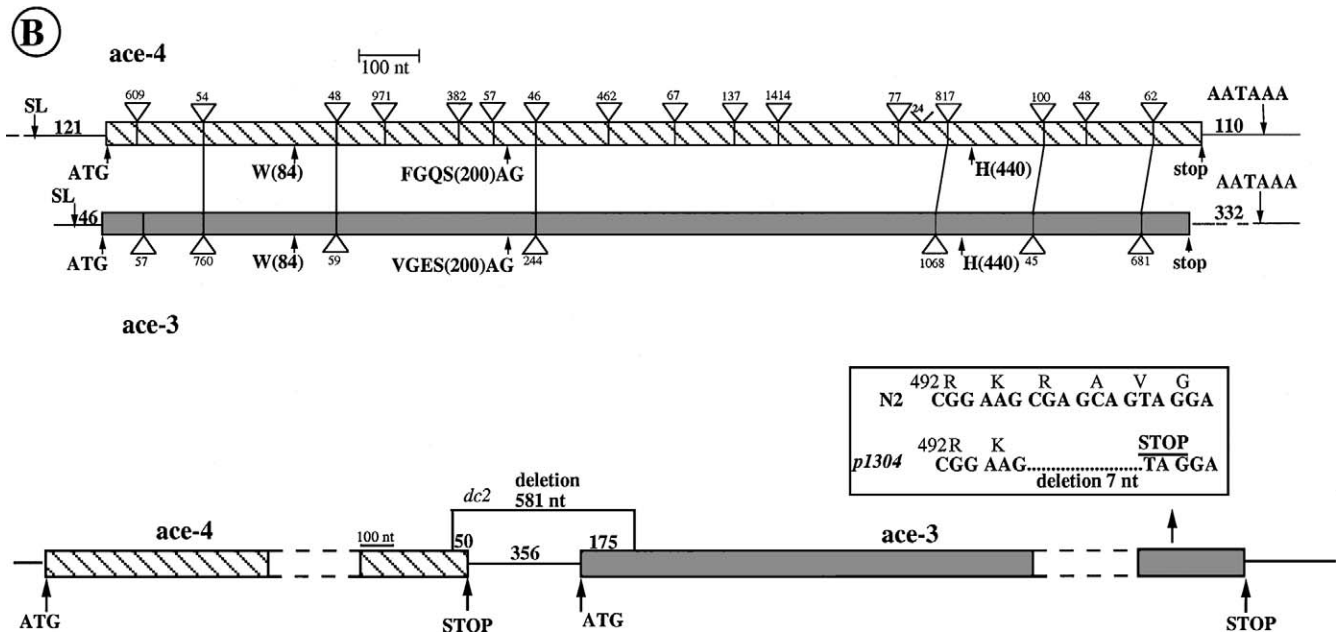
## III. Structure of the Four *ace* Genes

### A. Comparison of Gene Structures

Genomic structures of *ace* genes in *C. elegans* is shown in Fig. 3A, in comparison to *Torpedo* and *Drosophila* AChE genes. Whereas the splicing of two alternative exons in a single AChE gene of vertebrates gives H and T transcripts, one invertebrate *ace* gene gives either the H or the T transcript (Fig. 3A). There are 9 introns in *ace-1,* 8 in *ace-2,* 7 in *ace-3,* and 16 in *ace-4*. Three splicing sites in the C-terminal region are conserved in all *ace* genes of *Caenorhabditis* and also

FIG. 3 Genomic structure of *ace* genes. (A) Location of introns was compared on an alignment of coding sequences including *C. elegans ace* sequences as well as *Torpedo* and *Drosophila* AChEs. Note the conservation of only two splicing sites between invertebrate and vertebrate AChE genes: the site of alternative splicing in vertebrates and the preceding one (vertical arrows). In invertebrates, a third splicing site is conserved 11–13 amino acids upstream of the H440 of the catalytic triad. Note that among the unusually large number of introns in *ace-4,* introns 2, 3, and 7 (in addition to introns 13, 14, and 16) are found in conserved position in *ace-3*. Introns 8 and 10 of *ace-4* are found in conserved position with introns 3 and 4 in *Drosophila* but not with introns of other *C. elegans ace* genes. It is also noted that the site of the hydrophilic insertion in *Drosophila* (Hall and Spierer, 1986) precisely corresponds to the location of intron 3 in *ace-3* and *ace-4*. Introns of *ace* genes were also located on a reconstituted secondary structure of *ace-1*. We found that only 5 of the 40 splicing sites in *ace* genes interrupt segments encoding $\alpha$ helices (these sites are shown by an asterisk), and only 2 interrupt segments encoding $\beta$ sheets in secondary structures (triangles). Interestingly, most of those introns were found in *ace-4* (introns 4, 5, 11, 12, and 15). For each *ace* gene, the location of splicing sites is identical in *C. elegans* and *C. briggsae*. Location of introns in *Drosophila* and *Torpedo* AChE genes was taken from Fournier *et al.* (1989) and from Maulet *et al.* (1990). (B). Tandem organization of *ace-3* and *ace-4*. *N2 strain* (wild type): The genomic region covering *ace-4* (upstream gene) and *ace-3* (downstream gene) in N2 strain of *C. elegans* is drawn to scale except for introns. There are only 356 nt between the stop codon of *ace-4* and the initiator ATG of *ace-3,* and 200 nt between the polyadenylation site (AATAAA) of *ace-4* and the *trans*-splicing site (SL) of *ace-3*. In *C. briggsae, ace-4* and *ace-3* are separated by 369 nt. *Mutants:* Deletions in p1304 and dc2 strains are shown on a scheme of *ace-3* and *ace-4*. The p1304 mutation results in a truncated protein at K493 in *ace-3* (see box). dc2 is a large deletion of 581 nt removing the 3′ end of *ace-4,* the intergenic region, and the 5′ end of *ace-3*. (Adapted from D. Combes, Y. Fedon, M. Grauso, J.-P. Toutant, and M. Arpagous. Four genes encode acetylcholinesterases in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. cDNA sequences, genomic structures, mutation and *in vivo* expression. *Journal of Molecular Biology* 2000; 300:732–733.)


A
ace-1
(9 introns)
ace-2
(8 introns)
ace-3
(7 introns)
ace-4
(16 introns)
Drosophila
(8 introns)
Torpedo
(3 introns)
ATG
STOP
W*
S*
E*
H*
100 nt
exon H
exon T


B
ace-4
100 nt
SL
121
609
54
48
971
382
57
46
462
67
137
1414
77
24
817
100
48
62
AATAAA
110
ATG
W(84)
FGQS(200)AG
H(440)
stop
SL
146
AATAAA
332
ATG
57
760
W(84)
59
VGES(200)AG
244
1068
H(440)
45
681
stop
ace-3
492 R K R A V G
N2 CGG AAG CGA GCA GTA GGA
492 R K STOP
p1304 CGG AAG.....TA GGA
deletion 7 nt
dc2
deletion
581 nt
ace-4
100 nt
50
356
175
ace-3
ATG
STOP
ATG
STOP

in *Drosophila* AChE. Two of these sites are also conserved in *Torpedo* AChE. In particular, the site of alternative splicing in *Torpedo* AChE (and in mammalian AChEs), which leads to H or T transcripts, is conserved in all *ace* genes as well as in the *Drosophila* AChE gene. For each *ace* gene, the location of splicing sites is identical in *C. elegans* and *C. briggsae*. Except in *ace-4,* introns do not usually interrupt segments encoding units of secondary structure, but are found preferentially in loops or in unstructured regions. We found that only 5 of the 40 splicing sites in the *C. elegans ace* genes interrupt segments encoding $\alpha$ helices (these sites are shown by an asterisk), and only 2 interrupt segments encoding $\beta$ sheets in secondary structures (triangles). Interestingly, most of these introns were found in *ace-4* (introns 4, 5, 11, 12, and 15).

*C. elegans* introns have been shown to be much shorter than in vertebrates, with more than half of them being shorter than 60 nt (data based on an analysis of 669 *C. elegans* introns, Blumenthal and Steward, 1997). Of the 40 introns found in the four *ace* genes (see Table III), only 12 (30%) are shorter than 60 nt. They are often far larger (mean length of 378 nt, $n = 40$), and some are even exceptionally long (14/40 are longer than 500 nt). Table 3 shows also that two long introns are found in *ace-1, ace-2,* and *ace-3,* one located preferentially at the 5′ end (first or second splice site), and the other at the position of the conserved splice sites at the 3′ end (Table III). In *C. briggsae,* homologous introns tend to be shorter than in *C. elegans* (Blumenthal and Steward, 1997). Fourteen homologous introns in *ace-1, ace-2,* and *ace-3* are longer in *C. elegans* but 9 are longer in *C. briggsae*. The reverse situation is found in *ace-4,* where, of 16 homologous introns, 11 are longer in *C. briggsae*. Long introns sometimes contain interesting features, including other genes (for instance, *unc-17* in the first intron of *cha-1,* Alfonso *et al.,* 1994) or binding sites for transcription factors (Xue *et al.,* 1992). We thus looked at sequence conservation between homologous introns of *ace* genes, for identifying potentially functional elements in noncoding regions that otherwise have diverged extensively (Kennedy *et al.,* 1993).

## B. Analysis of Noncoding Regions in Ace Genes

Dot plot analysis is a powerful tool for identifying conserved sequences. As an example, a comparison of genomic sequences encompassing the whole coding sequences of *ace-1* in *C. elegans* and *C. briggsae* is shown in Fig. 4A. Exons are almost fully conserved between the two species and appear as bars. Introns are not conserved and appear as gaps. Conserved sequences were found only in intron 5 of *ace-4*. No sequence homology was found between other homologous introns of all *ace* genes, except for the short 5′ and 3′ borders that follow the consensus sequences for donor and acceptor splice sites (Grauso *et al.,* 1996).

TABLE III

Number, Location, and Length (in nt) of Introns in *ace* Genes in *C. elegans* and *C. briggsae*

| No. of introns in *ace-1* | *ace-1* | | *ace-2* | | *ace-3* | | *ace-4* | |
|---|---|---|---|---|---|---|---|---|
| | *C. elegans* | *C. briggsae* | *C. elegans* | *C. briggsae* | *C. elegans* | *C. briggsae* | *C. elegans* | *C. briggsae* |
| 1 | 818* | 56 | | | | | | |
| | | | | | 57 | 95 | 609 | 64 |
| | | | 879* | 985* | | | | |
| | | | | | 759* | 207* | 54 | 106 |
| 2 | 47 | 50 | | | | | | |
| | | | 104 | 42 | | | | |
| | | | | | 59 | 51 | 48 | 57 |
| | | | | | | | 971 | 1230 |
| 3 | 356 | 63 | | | | | | |
| | | | 255 | 44 | | | 382 | 403 |
| | | | | | | | 57 | 77 |
| | | | | | 244 | —[b] | 46 | 55 |
| 4 | 292 | 91 | | | | | | |
| | | | 46 | 48 | | | | |
| | | | | | | | 462 | 363 |
| 5 | 318 | 45 | | | | | | |
| | | | | | | | 67 | 131 |
| | | | 885 | 46 | | | | |
| | | | | | | | 136 | 200 |
| | | | | | | | 1414 | 2308 |
| | | | | | | | 77 | 91 |
| 6[a] | 1420** | 108 | 525** | 49 | 536** | 1770** | 817 | 118 |
| 7[a] | 45 | 58 | 513(**) | 48 | 45 | 59 | 100 | 83 |
| | | | | | | | 48 | 298 |
| 8[a] | 681(**) | 50 | 152 | 489** | 681(**) | 49 | 62 | 56 |
| 9 | 46 | 48 | | | | | | |
| Total introns | 3910 | 569 | 3359 | 1751 | 2381 | 2231 | 5350 | 5640 |
| Total exons | 1860 | 1860 | 1887 | 1887 | 1820 | 1812 | 1806 | 1812 |

*Note:* Numbering of introns (left column) is that of introns in *ace-1*.

[a] Introns 6, 7, and 8 in *ace-1* are located at the three splicing sites conserved in all invertebrate ace genes (see Fig. 3A). Only at those sites introns are drawn on the same line. The other splicing sites are not homologous in *ace* genes. In *ace-1, ace-2,* and *ace-3,* a very long intron is found at the 5′ end (*) and one at the 3′ end (**).

[b] There is no intron in *ace-3* of *C. briggsae* at this location.

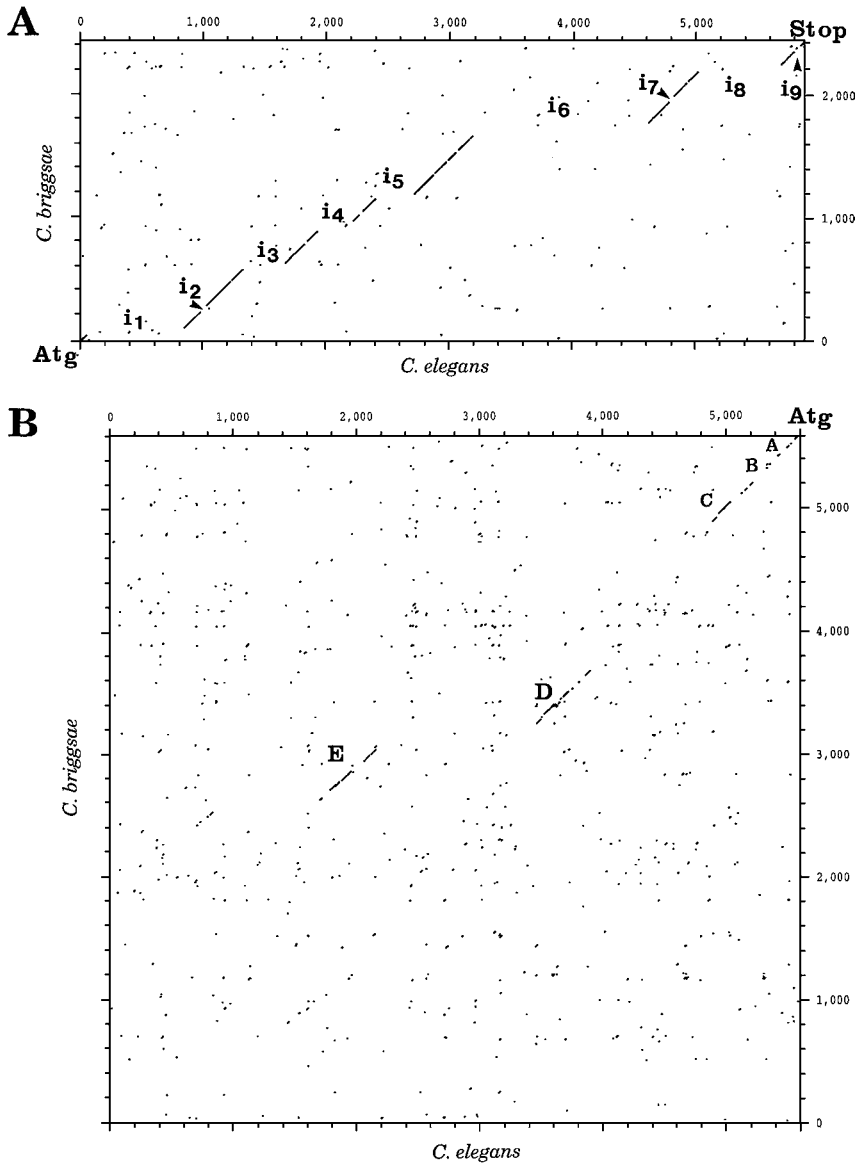



FIG. 4 Analysis of sequences conservation between *C. elegans* and *C. briggsae* by DotPlot analysis. For this type of experiment, we used Compare and DotPlot software from the Wisconsin Genetics Computer Group. (A) Comparison of genomic sequences of *ace-1* from the ATG (bottom left) to the STOP codons (top right). The comparison extends on 2432 nt in *C. briggsae* (*y* axis) and 5892 nt in *C. elegans* (*x* axis). Introns in *ace-1* are numbered as in Table III. Note the conservation in exons (bars) and the lack of conservation in introns (gaps). Shifts to the right are due to the larger sizes of introns in *C. elegans* than in *C. briggsae* (see Table III). (B) Comparison of 5′ flanking regions of *ace-1*. The comparison extends on 5600 nt on *C. elegans* (*x* axis) and *C. briggsae* (*y* axis) upstream of the ATG (top right corner). The comparison identifies blocks A, B, C, and D in the first 2.6 kb (Culetto *et al.,* 1999). An additional block E appears within 4 kb.

Dot plot analysis of 2.5 kb of the 5′ region of the *ace-1* gene in the two nematode species revealed the existence of four blocks of conserved sequences (A, B, C, and D), some of them driving the tissue-specific expression of the gene (see below and Culetto *et al.,* 1999). Figure 4B shows an extension of this analysis to 4 kb of 5′ regions in *ace-1* of both species: *elegans* and *briggsae*. An additional block of conserved sequence is identified (E). A similar comparison of 4 kb of homologous 5′ flanking regions was performed with *ace-2,* which revealed three blocks of conserved sequences located within 600 nt upstream of the ATG. The comparison of 2.5 kb of 5′ region upstream of *ace-4* (possible promoter region of the tandem *ace-3; ace-4*) showed two blocks of conserved sequences between *C. elegans* and *C. briggsae*. The role of these conserved sequences (intronic or 5′ regions) will be tested with GFP reporter genes.

### C. Tandem Organization of *Ace-3* and *Ace-4*

A scheme of the tandem organization of *ace-3* and *ace-4* is shown in Fig. 3B with the location of the two deletions found in mutants *ace-3*(p 1304)II and *ace-3*(dc2)II (see section II.D.2, above). In the N2 strain both *ace-3* and *ace-4* genes are first transcribed as a long, bicistronic mRNA which is further cleaved into individual messengers (see IV.B, below).

## IV. Expression of Ace Genes

### A. Transcription *in Vivo*

RT-PCR experiments were positive for the four *ace* genes, indicating that they are all transcribed *in vivo* (Grauso *et al.,* 1998). A direct assessment of transcripts abundance was achieved by Northern blots of RNAs extracted from a mixed-staged population. The abundance of *ace-1, ace-2,* and *ace-3* was essentially comparable with only a few variations in sizes (between 2.6 and 3.0 kb). Under these conditions, *ace-4* messenger RNAs were present but almost undetectable (Combes *et al.,* 2000).

### B. Trans-splicing

Most *C. elegans* transcripts are *trans*-spliced shortly after transcription (Krause and Hirsch, 1987; Zorio *et al.,* 1994; see Blumenthal and Steward, 1997, for a review). In *trans*-splicing, a post transcriptional event that exists in trypanosomes and nematodes, a short leader sequence (SL) is spliced to the 5′ end of messenger

RNAs. *Trans*-splicing could enhance translational efficiency (Maroney *et al.,* 1995). There are two types of spliced leader in *C. elegans,* SL1 and SL2, both 22 nt long. We checked whether transcripts of the four *ace* genes were *trans*-spliced by PCR using a reverse primer in the coding sequence and either SL1 or SL2 as sense primers. All PCR products were fully sequenced. We found that *ace-1* was *trans*-spliced to SL1 only (in agreement with Culetto *et al.* (1999) whereas *ace-2, ace-3,* and *ace-4* were *trans*-spliced to both SL1 and SL2 (Fig. 5; see color insert) So far, all genes *trans*-spliced to SL2 were found located within polycistronic units (operons), in a downstream position. The mean distance separating genes in *C. elegans* operons is very short (10 to 400 bp; review in Blumenthal and Steward, 1997). We thus looked at the position of the next genes either upstream or downstream of *ace-2, ace-3,* and *ace-4*. No other gene was found in the immediate vicinity of *ace-2,* upstream of *ace-4,* or downstream of *ace-3* (Combes *et al.,* 2000). It is thus possible that genes that do not belong to operons can be *trans*-spliced to SL2. Alternatively, *ace-2* or the tandem *ace-3; ace-4* could be downstream genes in nonconventional operons with unusually large intergenic regions.

We have shown recently that in wild-type *C. elegans* (N2), it is possible to detect by RT-PCR a bicistronic transcript containing both *ace-3* and *ace-4* in addition to the individual *trans*-spliced transcripts of *ace-3* and *ace-4* (Didier Combes and Martine Arpagaus, unpublished results). This shows that *ace-3* and *ace- 4* belong to an operon, sharing the same promoter. Polycistronic RNAs are usually extremely difficult to detect because they are processed very rapidly into individual mRNAs (see discussion in Blumenthal and Steward, 1997). So far we do not know why the bicistronic *ace-3; ace-4* RNA accumulates. We noted that the bicistronic RNA is usually not completely *cis*-spliced. In particular, the last intron of *ace-4* is often present, with or without the first intron of *ace-3*.

## C. Tissue-Specific Expression

### 1. Methods

5′ Regions sufficient to rescue double mutants *ace-1; ace-2* by *ace-1* or *ace- 2* genes was first determined. These regions were then placed upstream of the GFP reporter gene (Chalfie *et al.,* 1994) in a plasmid vector (gift of Dr. A. Fire, Carnegie Institute, Washington, DC) and injected into the gonad of N2 hermaphrodites (Mello *et al.,* 1991). The GFP vector was coinjected with the *rol-6* plasmid which confers a characteristic roller phenotype to the transformants. Several stable transformant lines were established for each construct. The tissue distribution of GFP fluorescence was then studied under the microscope in order to establish the expression pattern of *ace-1* and *ace-2*. Further details concerning the transformation technique can be found in Mello and Fire (1995).

## 2. Results

When GFP expression was driven by the *ace-1* promoter region, fluorescence was observed in all body wall muscle cells (Fig. 6a; see color insert). The twisted aspect of the muscle quadrants is due to the injection of the *rol-6* gene. In the head region, the three pharyngeal muscle cells pm5 were fluorescent, as were three pairs of sensory neurons (Fig. 6b; see color insert) identified as the two outer lateral labial neurons and the four sensory cephalic neurons CEP (Culetto *et al.,* 1999). Four of the eight vulval muscle cells, vm1, of the hermaphrodite were also labelled (Fig. 6c; see color insert) as well as the diagonal and spicule muscles of the male and the anal sphincter muscle (not shown). The same pattern of expression was observed when the 5′ region of *ace-1* of *C. briggsae* was used to transform *C. elegans* (Didier Combes, unpublished).

In contrast with *ace-1,* the fluorescence driven by *ace-2* was limited to the head and tail ganglion neurons, the nerve cord, and a few cells of the tail (Fig. 6d; see color insert). Note that there are several nerve processes extending to the extreme tip of the head (Fig. 6e; see color insert). The identification of nerve cells expressing *ace-2* is in progress.

We transfected some hermaphrodites with GFP under the control of 4 kb of the region upstream to *ace-4*. We observed GFP expression in only a few cells of the head, which have not been identified yet.

## 3. Effects of Promoter Deletions on *ace-1*::GFP Expression

*ace-1* expression seems to be regulated in a simple fashion by a basal promoter regulated by distinct tissue-specific activator elements located further upstream. At least blocks C and D were shown to be responsible for expression in body-wall and pharyngeal muscle cells, respectively (Culetto *et al.,* 1999). Figure 7 shows the lack of fluorescence in body-wall muscle cells after deletion of block D (compare with Figs. 6a and 6b).

## 4. Conclusions

In an analysis of mosaic animals where only some cells carry the *ace-1* gene, Herman and Kari (1985) concluded that *ace-1* expression was required in muscle cells but not in motor neurons for normal locomotion. Our GFP experiments support this initial observation: *ace-1* is prominently expressed in muscle cells and is not expressed in motor neurons. In addition our results suggest that *ace-2* is expressed in neurons (sensory and motor) but not in muscle cells. It is tempting to compare this situation to that observed in vertebrate neuromuscular junctions where a type H subunit, resembling *ace-2* (at least for the C-terminus, see below) is expressed by nerve cells and a T subunit, resembling *ace-1,* is expressed in muscle cells. It will be interesting to look at the precise repartition of both enzymes at the neuromuscular junctions in *C. elegans* (which have a particular structure;

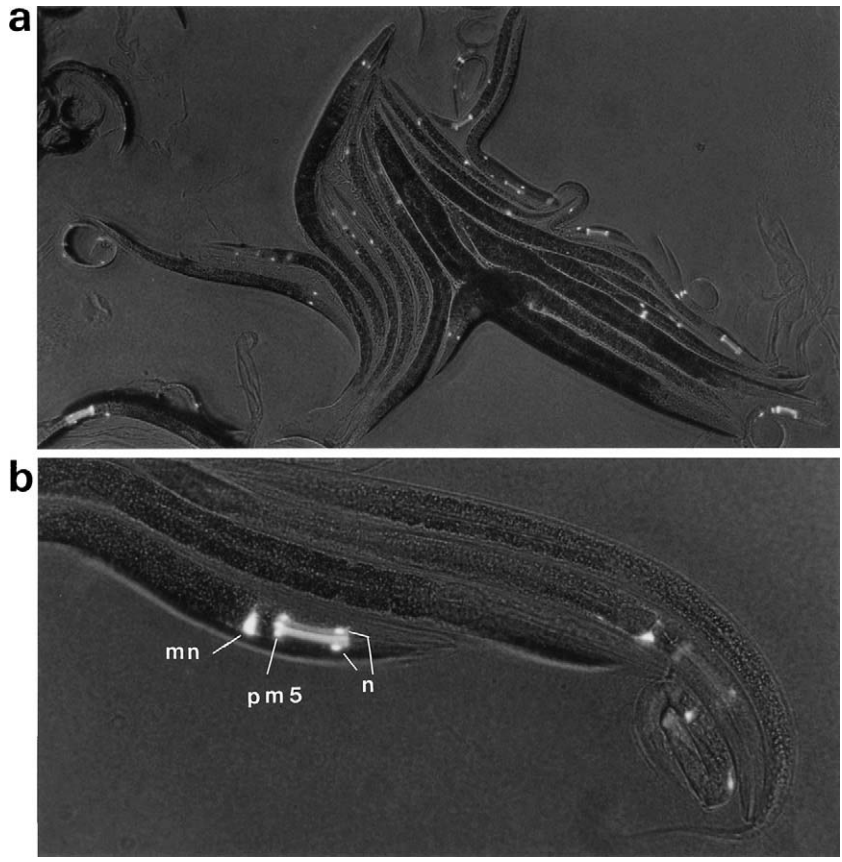



FIG. 7 Effect of deletion of block D on the expression of *ace-1*::GFP. Note the absence of fluorescence in body-wall muscle cells (a), which is restricted (b) to mn, head mesodermal cell; pm 5, pharyngeal muscle cells 5; and n, sensory neurons.

see White *et al.,* 1986, and Jorgensen and Nonet, 1995). At the moment, one can simply suppose that both muscle cells and neurons provide AChE at the synapse, explaining the lack of gross phenotype in *ace-1* or *ace-2* null mutants.

## V. The Four AChE Genes Products

### A. Pharmacological Properties

A straighforward kinetic and pharmacological analysis of both ACE-1 and ACE-2 was reported by Johnson and Russell (1983). They measured a $K_m$ of 1.2–1.5 $\times$ $10^{-5}$

M ACh for ACE-1 and of 7–8.0 $\times 10^{-5}$ M for ACE-2, two values which indicate similar or stronger affinities than vertebrate AChE and BChE. Both ACE-1 and ACE-2 hydrolyze butyrylcholine (BCh), as is the case for most invertebrate AChEs. The ratio of BCh/ACh hydrolysis (at 1 mM) was found to be 0.60 for ACE-1 and 0.2 for ACE-2. ACE-1 was found to be more sensitive to eserine than ACE-2 ($IC_{50}$: $3 \times 10^{-9}$ M and $2 \times 10^{-7}$ M, respectively) and this was also the case for edrophonium ($5 \times 10^{-7}$ M versus $10^{-5}$ M). ACE-1 and ACE-2 had approximately the same sensitivity to the organophosphate di-isopropylfluorophosphate (DFP, $10^{-5}$ M). ACE-1 is reversibly inhibited by the nonionic detergent Triton X-100, whereas ACE-2 has a particular sensitivity to the ionic detergent deoxycholate. We have shown recently that the sensitivity to gallamine and propidium, two ligands of the peripheral site that inhibits AChEs through allosteric interactions, ranged in the same order: ACE-1 was found to be more sensitive than ACE-2 and ACE-3 was almost totally resistant (Combes *et al.,* 2000).

ACE-3 was originally identified in double null mutants *ace-1; ace-2* (Kolson and Russel, 1985a). It represents only 5% of the total AChE activity in wild-type animals. Its $K_m$ for ACh ($1.6–1.8 \times 10^{-8}$ M) was 1000- and 5000-fold lower than that of ACE-1 and ACE-2, respectively. This explains that ACE-3 is still active at very low concentrations of ACh, at which ACE-1 and ACE-2 have no activity. ACE-3 hydrolyzes BSCh as well as ASCh, and another major characteristic is its high resistance to usual inhibitors of AChEs: in particular ACE-3 is about 3000-fold more resistant to eserine than ACE-2 and 260,000-fold than ACE-1 (Kolson and Russell, 1985b). This is also the case with the nematicide aldicarb used in agricultural practice. The molecular basis of this insensitivity is unknown for the moment.

Stern (1986) reported the existence of a fourth class of AChE in *C. elegans* which represents only 0.1% of the total AChE activity *in vivo*. $K_m$ of this class D AChE was shown to be $10^{-6}$ M ACh and it was eserine-resistant like ACE-3.

It is interesting to note that ACEs from *C. elegans* all hydrolyze significant amounts of butyrylthiocholine (BSCh) as well as ACh (or ASCh). ACE-3 is even faster on BSCh than on ASCh (Combes *et al.,* 2000). Accomodation of choline esters with long acyl chains seems to be a general property of all invertebrate AChEs studied so far, whereas vertebrate AChEs do not hydrolyze BSCh. This has been related to the size of the acyl pocket in the active site, which was believed to be larger in invertebrate AChEs than in vertebrate AChEs. Mutating the two residues F288 and F290 of the acyl pocket to nonaromatic residues allowed vertebrate AChEs to hydrolyze BSCh (Harel *et al.,* 1992; Vellom *et al.,* 1993). Interestingly alignments showed that position 288 was never occupied by an aromatic amino acid in invertebrates (see Table I). In vertebrate AChEs, residues 288 and 331 of the acyl pocket are both phenylalanines (Table I) that form a rigid $\pi$–$\pi$ stacking pair, whereas the presence of nonaromatic residues at position 288 in invertebrate

AChEs renders F/W331 more mobile, thus enabling to accomodate larger acyl moities (Harel *et al.,* 2000).

## B. Molecular Forms

Molecular forms (i.e., size isomers, see Massoulié and Toutant, 1988) have been identified by a combination of ultracentrifugation in 5–20% sucrose gradients and nondenaturing electrophoreses as originally described for the nematode *Steinernema carpocapsae* (a rhabditidae that possesses a high AChE activity; Arpagaus *et al.,* 1992).

### 1. ACE-1

The major molecular form produced by *ace-1 in vitro* (Sf9 cells infected by a recombinant baculovirus or transfected S2 *Drosophila* cells) is a tetramer which sediments at 11.5 S with smaller amounts of a monomer (4.5–5 S; see Fig. 8A). *In vivo* it is possible to study ACE-1 molecular forms in the mutants *ace-2*. We repeatedly found a major peak sedimenting at 13 S with small amounts of monomer. The "heavy" 13 S form was transformed into a 11.5 S form by mild proteolysis by proteinase K (Fig. 8B) and was partially reduced into monomers by DTT (Fig. 8C). We suggest that the 13 S form is a heterotetramer in which a noncatalytic subunit is associated to four catalytic components. This noncatalytic element could mediate the attachment of AChE to the external face of the membrane *in vivo* and should not be synthesized in *in vitro* expression systems (*ace-1* expressed in Sf9 cells of *Spodoptera* also gives a 11.5 S form; Arpagaus *et al.,* 1994). It is interesting to note that, in vertebrates, AChE G4 form includes such a noncatalytic component (P subunit) that interacts with the C-terminal portion of four T subunits (Bon *et al.,* 1997; Simon *et al.,* 1998). The relationship between P and the protein interacting with the C-terminal domain of ACE-1 in *C. elegans* is currently being studied.

### 2. ACE-2

We used the mutant *ace-1* (strain *ace-1*(p 1000)X) to further characterize ACE-2. In a sucrose gradient containing Triton X-100 (not shown), there is a major 6.5 S peak (dimeric form, $G_2$) which is shifted to approximately 3 S in the presence of Brij 96, indicating its amphiphilic character (Fig. 9, amphiphilic dimer or $G_2$a). Variable amounts of hydrophilic $G_2$ (6.7–7 S) and of hydrophilic $G_1$ (4 S) are also present. PI-PLC converted the 3 S form into a 7 S hydrophilic dimer (Fig. 9), showing that the $G_2$a form possesses a glycolipid anchor. The inset in Fig. 9 shows that $G_2$a recovered after a preparative centrifugation was converted by PI-PLC into a hydrophilic, fast-migrating dimer. This behavior is typical of amphiphilic dimeric AChE of type I (Bon *et al.,* 1988).

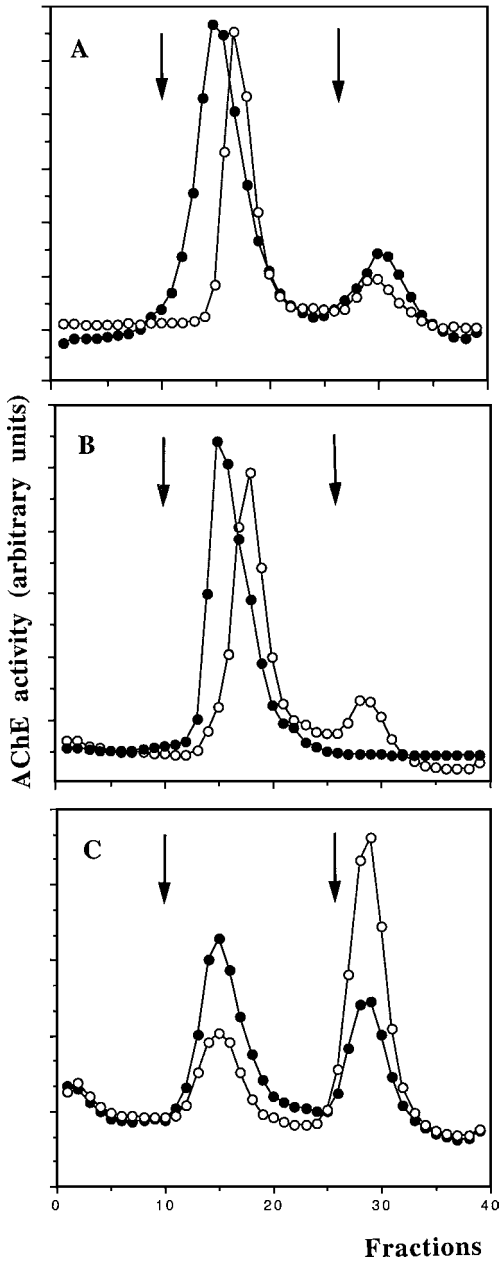



FIG. 8 Molecular forms of ACE-1. (A) AChE was extracted from an *ace-2* mutant (strain *ace-2* (g72)I) using a high salt buffer (HS) containing 0.5% of Brij 96 (HSB) and analyzed on a 5–20% sucrose gradient in HS (filled circles). The profile is compared to the molecular forms produced in S2 cells (Schneider cells from *Drosophila*) transfected by a vector containing the full coding sequence of *ace-1*. Transformed cells were extracted using HSB buffer and the extract was analyzed on HS gradients (open circles). There is a 5 S (monomeric) form (G1) in both cases as well as a major 13 S form *in vivo*

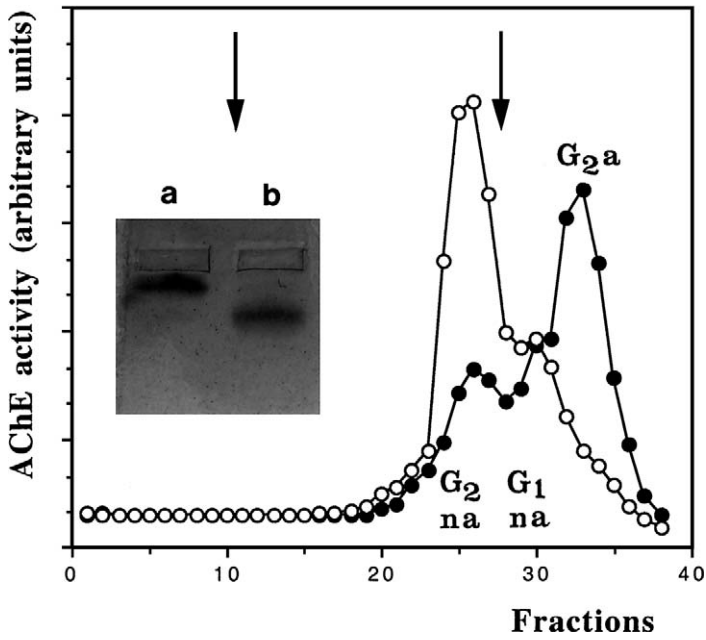



FIG. 9 Molecular foms of ACE-2. Ultracentrifugation of a HSB extract of *ace-1* strain (ACE-2) on HSB gradient (filled circles). Hydrophilic dimers ($G_2$na) and monomers ($G_1$na) sediment at 6.7–7.0 S and 4.5 S (shoulder), respectively, and the amphiphilic dimers ($G_2$a) sediment at 3.0 S. After treatment by PI-PLC (open circles), the 3 S peak is converted into a hydrophilic, 7.0 S form ($G_2$a of type I). Vertical arrows: left, position of $\beta$-galactosidase (16 S), and right, alkaline phosphatase (6.1 S) (Inset) PI-PLC susceptibility of ACE-2 in nondenaturing electrophoresis. AChE activity was stained according to Karnovsky and Roots (1964). Lane a, control sample; lane b, treated by PI-PLC from *Bacillus cereus* (final concentration of 5 units/ml) for 1 h 30 min at 20°C. Increase in mobility indicates cleavage of the hydrophobic domain by PI-PLC. (Reproduced with permission from D. Combes, Y. Fedon, M. Grauso, J.-P. Toutant, and M. Arpagous. Four genes encode acetylcholinesterases in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. cDNA sequences, genomic structures, mutation and *in vivo* expression. *Journal of Molecular Biology* 2000;300:736.)

---

(heterotetramer; see text) or 11.5 S form *in vitro* (homotetramer). (B) Effect of proteinase K on the 13 S form produced by *C. elegans in vivo*. Top fractions of the 13 S peak were recovered on preparative gradients similar to that shown in A and treated with a final concentration of 25 $\mu$g/ml of Proteinase K for 1 h at 20°C (open circles). Note the shift from 13 S (control, filled circles) to 11.5 S (open circles). (C) Effect of reduction of disulfide bonds by DTT. A HSB extract of *ace-2* mutants was treated by $5 \times 10^{-3}$ M DTT for 1 h at 37°C (pH 8) in the presence of edrophomium for protecting the active site and then incubated with $10^{-2}$ M NEM for 15 min, dialyzed extensively, and analyzed on a HS gradient (open circles). Note partial conversion of the 13 S form into the 5 S form. Control sample (filled circles) was treated similarly except that DTT was omitted during the 1-h incubation at 37°C. Vertical arrows show the location of commercial $\beta$-galactosidase (16 S, left) and alkaline phosphatase (6.1 S, right) used as internal markers. Thirty-nine fractions were collected from each sucrose gradient and assayed with the Ellman reaction (Ellman *et al.,* 1961; see Arpagaus *et al.,* 1992, for complete description of the methods).

### 3. ACE-3 and ACE-4

We analyzed ACE-3 by using a high w/v ratio for extraction of *ace-1; ace-2* double mutants, and loading as much as 500 $\mu$l of extract on sucrose gradients. ACE-3 sedimented as a 7 S form, interacting with nondenaturing detergents ($G_2$a). These interactions were suppressed by PI-PLC. In the experimental conditions used to study ACE-3, we failed to detect any other AChE activity that could be attributed to ACE-4.

In conclusion, a hypothetical scheme of the diversity of molecular forms in *C. elegans* is shown and discussed in Fig. 10. We assume that this work model could be adapted for other nematode species, sometimes with a reduced diversity when only one or two *ace* genes exist or are expressed (see below).

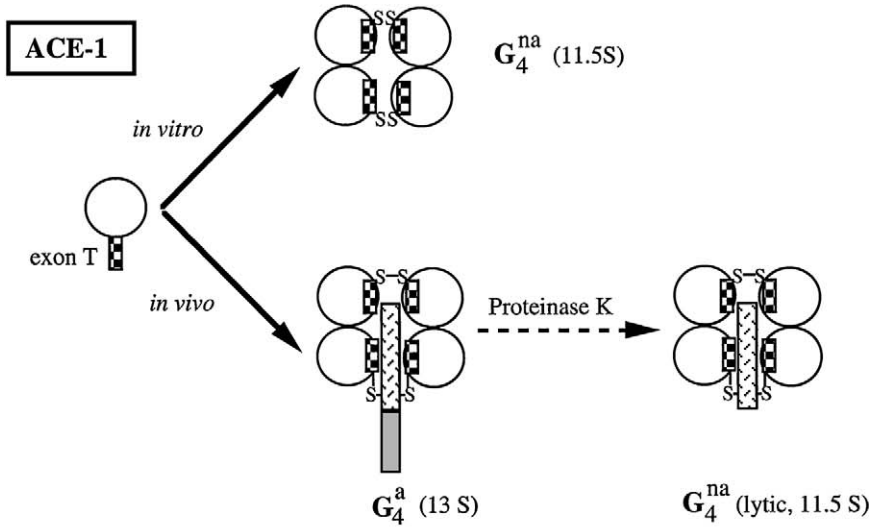
## VI. AChE Genes in Other Nematodes

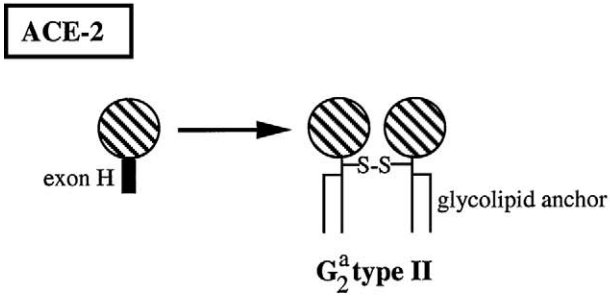
### A. Animal Parasites

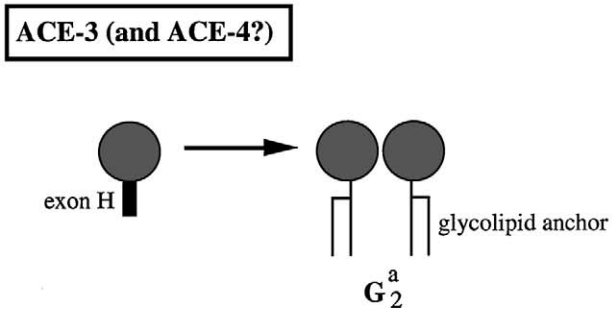
Two classes of AChEs were characterized in the rhabditidae *Steinernema carpocapsae,* a nematode parasite of insects (Arpagaus *et al.,* 1992). These AChEs exist as an amphiphilic heterotetramer and a glycolipid-anchored dimer resembling molecular forms of ACE-1 and ACE-2 of *C. elegans,* respectively. Although the corresponding genes were not sequenced in *Steinernema,* it is tempting to suggest that they are close to *ace-1* and *ace-2,* encoding a T and an H subunit, respectively.

---

FIG. 10 A schematic representation of molecular forms of each AChE class in the nematode *Caenorhabditis elegans*. The major molecular form of ACE-1 produced *in vivo* is drawn as a tetramer containing a structural subunit ("heterotetramer" in text). This structure resembles that of G4a form of vertebrate AChE (Bon *et al.,* 1997; Giles, 1997), but we assume that S–S bonds play a stronger role and that the structural component is heavier than in vertebrate AChEs, explaining the effect of DTT and the unusually high 13 S value. We show the interaction of the structural element with only two catalytic subunits but it could interact with four (see Bon *et al.,* 1997; Simon *et al.,* 1998 and Massoulié *et al.,* 1998). Proteinase K digestion is supposed to cleave off a substantial portion of the structural element, including the zone responsible for hydrophobic interactions, and gives the 11.5 S "lytic" form. The G4 form (11.5 S) synthesized *in vitro* could be a tetramer without structural element ("homotetramer" in text). The amphiphilic character of the G1 form is conferred by the C-terminal T sequence which can adopt the configuration of an amphiphilic $\alpha$ helix (Giles *et al.,* 1997; Massoulié *et al.,* 1998). ACE-2 and ACE-3 (and possibly ACE-4) are G2a forms of type I (i.e., glypiated subunits) linked (ACE-2) or not linked (ACE-3, ACE-4) by an interchain disulfide bond (see Fig. 2). In the absence of interchain S–S bond, the dimer could be held together by the "four-helix bundle," involving two $\alpha$ helices from each subunit (Sussman *et al.,* 1991; Harel *et al.,* 2000).


ACE-1
in vitro
in vivo
exon T
S-S
$G_4^{na}$ (11.5S)
Proteinase K
$G_4^{a}$ (13 S)
$G_4^{na}$ (lytic, 11.5 S)


ACE-2
exon H
S-S
glycolipid anchor
$G_2^{a}$ type II


ACE-3 (and ACE-4?)
exon H
glycolipid anchor
$G_2^{a}$

In contrast, *Parascaris aequorum* possesses a single AChE characterized as an amphiphilic nonglypiated dimer (Talesa *et al.,* 1997). This apparent restriction in AChE variability could be related to the expression of a single gene which, in turn, could be related to the reduction of mobility in this parasite in comparison to the free living nematode *C. elegans*.

A remarkable phenomenon is observed with other nematodes that inhabit the gastrointestinal tract of mammalian hosts: some of them secrete large amounts of AChE when they are maintained *in vitro* and they are supposed to do so *in situ.* It is the case for *Necator americanus* and for *Trichostrongylus colubriformis,* two nematodes parasites of human and sheep, respectively (Pritchard *et al.,* 1991, 1994, Griffiths and Pritchard, 1994).

In *Nippostrongylus brasiliensis,* a nematode parasite of rat jejunum, the secreted AChE was purified and the N-terminal sequence was used for isolating a full-length cDNA clone (Hussein *et al.,* 1999a). The deduced sequence presents the conserved residues, W84, S200, E327, and H440, three conserved internal S–S bonds, and 11 of the 14 aromatic residues lining the catalytic gorge that define an AChE. Surprisingly, the cDNA ends near the location of the last splicing site in 3′ common to all AChEs (see Fig. 3A). The resulting protein thus possesses a short hydrophilic C-terminus. When expressed *in vivo* (Grigg *et al.,* 1997) or in the methylotropic yeast *Pichia pastoris* (Hussein *et al.,* 1999a), the AChE gene from *N. brasiliensis* produces a high level of hydrophilic monomer. These properties of the secreted *Nippostrongylus* AChE are similar to that secreted in the venom of the snake *Bungarus* (Cousin *et al.,* 1998a). Another pharmacological class of AChE was identified in somatic extracts of *Nippostrongylus.* This enzyme appears essentially under an amphiphilic tetrameric form (Hussein *et al.,* 1999b). The relationship between this somatic (neural?) AChE with the secreted form(s) of AChE in *Nippostrongylus* is currently being studied by the group of M. Selkirk (London, UK). It will be interesting to check the possible relationships between those AChE forms in *Nippostrongylus* and the different classes of AChE characterized in *C. elegans*.

The AChE secreted by parasitic nematodes could downregulate the processes of host-protective inflammation by inhibiting the activation of lymphoid or myeloid cells (Maizels *et al.,* 1993; Pritchard *et al.,* 1998) or/and could represent a response to the increased expression of muscarinic ACh-Rs in intestinal cells after the entry of parasites. Additional muscarinic ACh-Rs could increase the acetylcholine-mediated chloride and mucus secretion, two events that certainly contribute to the expulsion of pathogens. Large amounts of secreted AChE would reduce such response (Selkirk *et al.,* 1998).

## B. Phytoparasites

The root-knot nematode, *Meloidogyne,* possesses three classes of AChEs, A, B, and C, which can be compared to the homologous classes of AChE in *C. elegans,*

on the basis of substrate specificity, inhibitor, and nondenaturing detergent susceptibility and thermal inactivation (Chang and Opperman, 1991). Class B is absent in *Heterodera glycines,* which thus possesses a larger percentage of its total AChE as class C (relatively resistant to anthelminthic carbamates; Chang and Opperman, 1992).

A gene encoding an AChE in *Meloidogyne incognita* and *M. javanica* was cloned and sequenced (Piotte *et al.,* 1999). Those sequences present strong homologies with *ace-1* of *C. elegans* (see Table II), including the characteristic T sequence in the C-terminal part (T subunit; see Fig. 2). Northern blot analysis shows that *ace-1* is transcribed in eggs and juveniles prior to the host infestation but not in parasitic females and males (Piotte *et al.,* 1999).

## VII. Concluding Remarks and Perspectives

### A. Phylogeny of AChE

We started the study of AChE in invertebrates in 1984 at a moment where the gross biochemical structure of vertebrate AChE was already largely elucidated (Massoulié and Bon, 1982). At that time, the idea was that the structure of invertebrate AChE could well be totally different from that in vertebrates. In fact we rapidly identified a glypiated dimer as the single molecular form of insect AChE (Arpagaus and Toutant, 1985; Toutant, 1989, for a review), very close to the G2a of type I defined in vertebrates (Bon *et al.,* 1988; Massoulié and Toutant, 1988, for a review). In further studies of AChE in nematodes (first on *Steinernema* and then on *Caenorhabditis*) we showed that one gene (*ace-1*) gave rise to an amphiphilic tetramer of catalytic subunits resembling the G4a form of vertebrates and that *ace-2* and *ace-3* (and possibly *ace-4*) gave dimers of glypiated AChE subunits. However, in spite of these similarities with vertebrate AChEs that concern essentially the C-terminal part of the sequences, it is difficult to derive a simple hypothesis on molecular evolution of AChE. If one excludes the relatively recent duplication that led to *ace-3* and *ace-4* in the genus *Caenorhabditis,* the low percentages of identity between ACE-1, ACE-2, and ACE-3 (Table II) suggest that the existence of three distinct genes is a very ancient situation. In addition, there is no reason to establish a privileged relationship between one of the nematode AChE genes and the insect or vertebrate AChE genes (Table II). The analogy of structure in C-terminals (between *ace-1* and T subunits of vertebrate AChEs and between *ace-2, ace-3, ace-4,* and H subunits) could have resulted from an exon capture. It should be noted that the active serine 200 is encoded by TCn in all *ace* genes of *Caenorhabditis* and *Drosophila* but by AGy in vertebrate AChEs. Since TCn and AGy cannot interconvert by a single mutation, Brenner (1988) suggested a separate origin for invertebrate and vertebrate AChEs.

The multiplicity of *ace* genes in nematodes remains puzzling for the general phylogeny of AChE, especially if one considers that the condensed genome of *C. elegans* usually contains a lower number of genes in a given gene family than in vertebrates. It should be noted, however, that the family of ACh-R subunits is also larger in *C. elegans* than in vertebrates (David Sattelle, pers. commun.).

## B. *Ace-4*: An AChE Gene on Its Way of Elimination?

We have shown that *ace-4* (as the other *ace* genes) is transcribed and *trans*-spliced, but its messengers are in minute amounts and the corresponding AChE activity is not detected (Combes *et al.,* 2000). This strongly suggests that *ace-4* is a non functional AChE gene. When a gene is duplicated, one of the copies may become silenced by degenerative mutations, or one copy may acquire a novel beneficial function, or both copies may become partially compromised by accumulation of mutations (Lynch and Conery, 2000). The active site sequence in ACE-4 (FGQS*AG instead of the usual FGES*AG) explains the lack of enzymatic activity as shown by site-directed mutagenesis E199Q in vertebrate AChE (Taylor and Radic, 1994). *ace-4* did not, however, accumulate nucleotide substitutions as expected for a nonfunctional gene (Combes *et al.,* 2000). In addition, *ace-4* is also present in the genome since at least the estimated date of speciation of *C. elegans* and *C. briggsae* (40 Myr, Kennedy *et al.,* 1993), whereas the estimated half-life of elimination of nonfunctional extra genes is only 3 Myr in *C. elegans* (Lynch and Conery, 2000).

This could mean that ACE-4 has acquired another function, independent from the catalytic activity. We will address this question directly by inactivating *ace-4* (alone or in combination with *ace-3*) by RNA interference (Fire *et al.,* 1998).

## C. Conclusion

Nematode worms cause each year a worldwide crop loss of $80–100 billions. Helminth parasites are highly prevalent in human communities in developing countries and represent a severe threat to farm animals in developed ones. Anticholinesterase drugs are largely used against plant parasites and also sometimes against animal endoparasites. The hazards of these treatments come from the susceptibility of mammalian AChEs: incidental or chronic exposures of humans or domestic animals to anti-AChEs can have devastating consequences. Moreover, class C AChE which is naturally resistant to anti-AChE drugs, can represent a high proportion of total AChE activity in some species like *Heterodera glycines* (Chang and Opperman, 1992). As a consequence, these nematodes have a lower sensitivity to conventional treatments. The complete elucidation of the structure of

nematode AChEs, and particularly the 3D structure of the active sites is currently under way. This will hopefully boost the research of new anticholinesterase agents more specific for nematodes, which could be used more safely in fields. Such a development of new chemical agents might be, however, restricted on account of environmental concerns. Another strategy is the production of transgenic plants refractory to the infestation of worms. A resistance gene, whose mechanism of action is unknown, has been isolated from *H. glycines,* and the resistance can be transferred to sensitive species (Cai *et al.,* 1997). Plant genes that are upregulated during the early steps of formation of nematode feeding sites have been isolated (Opperman *et al.,* 1994; Favery *et al.,* 1998), and their promoters can be used to direct production of proteins toxic to the parasite such as proteinase inhibitors (anti-feedant approach, Lilley *et al.,* 1999).

## Acknowledgments

This work was supported by the *Institut National de la Recherche Agronomique,* the *Association Française contre les Myopathies* and by the Programme Quality of Life of the European Community (Project QLK3-CT-2000-00650, ACHEB). D.C. was a recipient of the *Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche.* We thank Drs. Marta Grauso and Emmanuel Culetto (now at the Department of Human Genetics, Oxford, UK), who were involved in the *C. elegans* work for several years here at Montpellier. We also appreciate the help of the *Caenorhabditis* Genetics Center (Saint Paul, MN), funded by the NIH National Center for Research Sources.

## References

Alfonso, A., Grundahl, J. S., McManus, J. M., and Rand, J. B. (1994). Alternative splicing leads to two cholinergic proteins in *Caenorhabditis elegans. J. Mol. Biol.* **241,** 627–630.

Arpagaus, M., Fedon, Y., Cousin, X., Chatonnet, A., Bergé, J.-B., Fournier, D., and Toutant, J.-P. (1994). cDNA sequence, gene structure, and *in vitro* expression of *ace-1,* the gene encoding acetylcholinesterase of class A in the nematode *Caenorhabditis elegans. J. Biol. Chem.* **269,** 9957–9965.

Arpagaus, M., Richier, P., Bergé, J.-B., and Toutant, J.-P. (1992). Acetylcholinesterases of the nematode *Steinernema carpocapsae*. Characterization of two types of amphiphilic forms differing in their mode of membrane association. *Eur. J. Biochem.* **207,** 1101–1108.

Arpagaus, M., and Toutant, J.-P. (1985). Polymorphism of acetylcholinesterase in adult *Pieris brassicae* heads. Evidence for detergent-insensitive and Triton-X-100-interacting forms. *Neurochem. Int.* **7,** 793–804.

Blumenthal, T., and Steward, K. (1997). RNA processing and gene structure. *In "C. elegans II"* (D. L. Riddle, T. Blumenthal, B. J. Meyer, and J. R. Priess, Eds.), pp. 117–145. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bon, S., Chang, J.-Y, and Strosberg, A. D. (1986). Identical N-terminal peptide sequences of asymmetric and of low-salt soluble and detergent-soluble dimers of *Torpedo* acetylcholinesterase. *FEBS Lett.* **209,** 206–211.

Bon, S., Coussen, F., and Massoulié, J. (1997). Quaternary associations of acetylcholinesterases. II. The polyproline attachment domain of the collagen tail. *J. Biol. Chem.* **272,** 3016–3021.

Bon, S., Toutant, J.-P., Méflah, K., and Massoulié, J. (1988). Amphiphilic and nonamphiphilic forms

of *Torpedo* cholinesterases. II. Electrophoretic variants and phosphatidylinositol phospholipase C-sensitive and -insensitive forms. *J. Neurochem.* **51,** 786–794.

Brenner, S. (1988). The molecular evolution of genes and proteins: A tale of two serines. *Nature* **334,** 528–530.

Bucht, G., and Hjalmarson, K. (1996). Residues in *Torpedo californica* acetylcholinesterase necessary for processing to a glycosyl phosphatidylinositol-anchored form. *Biochim. Biophys. Acta* **1292,** 223–232.

Cai, D., Kleine, M., Kifle, S., Harloff, H.-J., Sandal, N. N., Marcker, K. A., Klein-Lankhorst, R. M., Salentjin, E. M., Lange, W., Stiekema, W. J., Wyss, U., Grunder, F. M. W., and Jung, C. (1997). Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275,** 832–834.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., and Prasher, D. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

Chang, S., and Opperman, C. H. (1991). Characterization of acetylcholinesterase molecular forms of the root-knot nematode, *Meloidogyne. Mol. Biochem. Parasitol.* **49,** 205–214.

Chang, S, and Opperman, C. H. (1992). Separation and characterization of *Heterodera glycines* acetylcholinesterase molecular forms. *J. Nematol.* **24,** 148–155.

Combes, D., Fedon, Y., Grauso, M., Toutant, J.-P., and Arpagaus, M. (2000). Four genes encode acetylcholinesterases in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*. cDNA sequences, genomic structures, mutations and *in vivo* expression. *J. Mol. Biol.* **300,** 727–742.

Cousin, X., Bon, S., Massoulié, J., and Bon, C. (1998a). Identification of a novel type of alternatively spliced exon from the acetylcholinesterase gene of *Bungarus fasciatus. J. Biol. Chem.* **273,** 9812–9820.

Cousin, X., Hotelier, T., Giles, K., Toutant, J.-P, and Chatonnet, A. (1998b). aCHEdb: The database system for ESTHER: The $\alpha/\beta$ fold family proteins and the cholinesterase gene server. *Nucleic Acids Res.* **26,** 226–228.

Culetto, E., Combes, D., Fedon, Y., Roig, A., Toutant, J.-P., and Arpagaus, M. (1999). Structure and promoter ativity of the 5′ flanking region of *ace-1,* the gene encoding acetylcholinesterase of class A in *Caenorhabditis elegans. J. Mol. Biol.* **290,** 951–966.

Culotti, J. G., Von Ehrenstein, G., Culotti, M. R., and Russell, R. L. (1981). A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans. Genetics* **97,** 281–305.

Eisenbaher, B., Bork, P., Yuan, Y., Loeffler, G., and Eisenbaher, F. (2000). Automated annotation of GPI anchor sites: Case study, *C. elegans. Trends. Biochem. Sci.* **25,** 340–341.

Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterases activity. *Biochem. Pharmacol.* **7,** 88–95.

Favery, B., Lecomte, P., Gil, N., Bechtold, N., Bouchez, D., Dalmasso, A., and Abad, P. (1998). RPE, a plant gene involved in early developmental steps of nematode feeding cells. *EMBO J.* **17,** 6799–6811.

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature* **391,** 806–811.

Fournier, D., Karch, F., Bride, J. M., Hall, L. M. C., Bergé, J. B., and Spierer, P. (1989). *Drosophila melanogaster* acetylcholinesterase gene. Structure, evolution and mutations. *J. Mol. Biol.* **210,** 15–22.

Giles, K. (1997). Interactions underlying subunit association in cholinesterases. *Protein Eng.* **10,** 677–685.

Grauso, M., Culetto, E., Bergé, J.-B., Toutant, J.-P., and Arpagaus, M. (1996). Sequence comparison of *ace-1,* the gene encoding acetylcholinesterase of class A, in the two nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae. DNA Sequence* **6,** 217–227.

Grauso, M., Culetto, E., Combes, D., Fedon, Y., Toutant, J.-P., and Arpagaus, M. (1998). Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *C. briggsae. FEBS Lett.* **424,** 279–284.

Griffiths, G., and Pritchard, D. I. (1994). Purification and biochemical characterization of

acetylcholinesterase (AChE) from the excretory/secretory products of *Trichostrogylus colubriformis. Parasitology* **108,** 579–586.

Grigg, M. E., Tang, L., Hussein, A. S., and Selkirk, M. E. (1997). Purification and properties of monomeric (G1) forms of acetylcholinesterase secreted by *Nippostrongylus brasiliensis. Mol. Biochem. Parasitol.* **90,** 513–524.

Hall, L. M. C., and Spierer, P. (1986). The *Ace* locus of *Drosphila melanogaster:* Structural gene for acetylcholinesterase with an unusual 5′ leader. *EMBO J.* **5,** 2949–2954.

Harel, M., Kryger, G., Rosenberry, T. L., Mallender, W. D., Lewis, T., Fletcher, R. J., Guss, J. M., Silman, I., and Sussman, J. L. (2000). Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Sci.* **9,** 1063–1072.

Harel, M., Sussman, J. L., Krejci, E., Bon, S., Chanal, P., Massoulié, J., and Silman, I. (1992). Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. *Proc. Natl. Acad. Sci. USA* **89,** 10827–10831.

Herman, R. K., and Kari, C. K. (1985). Muscle-specific expression of a gene affecting acetylcholinesterase in the nematode *Caeorhabditis elegans. Cell* **40,** 509–514.

Hussein, A. S., Chacon, M., Smith, A. M., Tosado-Acevedo, R., and Selkirk, M. E. (1999a). Cloning, expression and properties of a nonneuronal acetylcholinesterase from the parasitic nematode *Nippostrongylus brasiliensis. J. Biol. Chem.* **274,** 9312–9319.

Hussein, A. S., Grigg, M. E., and Selkirk, M. E. (1999b). *Nippostrongylus brasiliensis:* Characterisation of a somatic amphiphilic acetylcholinesterase with distinct properties to the secreted enzymes. *Exp. Parasitol.* **91,** 144–150.

Johnson, C. D., Duckett, J. G., Culotti, J. G., Herman, R. K., Meneely, P. M., and Russell, R. L. (1981). An acetylcholinesterase-deficient mutant of the nematode *Caenorhabditis elegans. Genetics* **97,** 261–279.

Johnson, C. D., Rand, J. R., Herman, R. K., Stern, B. D., and Russell, R. L. (1988). The acetylcholinesterase genes of *C. elegans:* Identification of a third gene (*ace-3*) and mosaic analysis of a synthetic lethal phenotype. *Neuron* **1,** 165–173.

Johnson, C. D., and Russell, R. L. (1983). Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans. J. Neurochem.* **41,** 30–46.

Jorgensen, E. M., and Nonet, M. L. (1995). Neuromuscular junctions in the nematode *C. elegans. Semin. Dev. Biol.* **6,** 207–220.

Karnovsky, M. J., and Roots, L. (1964). A 'direct-coloring' thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12,** 219–221.

Kennedy, B. P., Aamodt, E. J., Allen, F. L., Chung, M. A., Heschl, F. P., and McGhee, J. D. (1993). The gut esterase gene (*ges-1*) from the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae. J. Mol. Biol.* **229,** 890–908.

Kolson, D. L., and Russell, R. L. (1985a). New acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans. J. Neurogenet.* **2,** 69–91.

Kolson, D. L., and Russell, R. L. (1985b). A novel class of acetylcholinesterase, revealed by mutations, in the nematode *Caenorhabditis elegans. J. Neurogenet.* **2,** 93–110.

Krause, M., and Hirsh, D. (1987). A *trans*-spliced leader sequence on actin mRNA in *C. elegans. Cell* **49,** 753–761.

Legay, C., Bon, S., and Massoulié, J. (1993). Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase. *FEBS Lett.* **315,** 163–166.

Lilley, C. J., Urwin, P. E., and Atkinson, H. J. (1999). Characterization of plant nematode genes: Identifying targets for a transgenic defence. *Parasitology* **118,** S63–S72.

Lynch, M., and Conery, J. S. (2000). The evolutionary fate and consequence of duplicate genes. *Science* **290,** 1151–1155.

Maizels, R. M., Don, A. P., Selkirk, M. E., Smith, D. F., and Anderson, R. M. (1993). Immunological modulation and evasion by helminth parasites in human populations. *Nature* **365,** 797–805.

Maroney, P. A., Denker, J. A., Darzynkiewics, E., Laneve, R., and Nilsen, T. W. (1995). Most

mRNAs in the nematode *Ascaris lumbricoides* are *trans*-spliced: A role for spliced leader addition in translational efficiency. *RNA* **1,** 714–723.

Massoulié, J., Anselmet, A., Bon, S., Krejci, E., Legay, C., Mayat, E., Morel, N., and Simon, S. (1998). Diversity and processing of acetylcholinesterase. *In* "Structure and Function of Cholinesterases and Related Proteins" (B. P. Doctor *et al.,* Eds.), pp. 3–24. Plenum Press, New York.

Massoulié, J., and Bon, S. (1982). The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Ann. Rev. Neurosci.* **5,** 57–106.

Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F.-M. (1993). Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* **41,** 31–91.

Massoulié, J., Sussman, J. L., Doctor, B. P., Soreq, H., Velan, B., Cygler, M., Rotundo, R., Shafferman, A., Silman, I., and Taylor, P. (1992). Recommendations for nomenclature in cholinesterases. *In* "Multidisciplinary Approaches to Cholinesterase Functions" (A. Shafferman and B. Velan, Eds.), pp. 285–288. Plenum Publishing, New York.

Massoulié, J., and Toutant, J.-P. (1988). Vertebrate cholinesterases: Structure and types of interaction. *Handbook of Expl. Pharmacol.* **86,** 167–224.

Maulet, Y., Camp, S., Gibney, G., Rachinsky, T., Ekström, T., and Taylor, P. (1990). Single gene encodes glycophospholipid-anchored and asymmetric acetylcholinesterase forms: Alternative coding exons contain inverted repeat sequences. *Neuron* **4,** 295–301.

Mello, C., and Fire, A. (1995). DNA transformation. *Methods Cell Biol.* **48,** 451–512.

Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans:* Extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10,** 3959–3970.

Opperman, C. H., Taylor, C. G., and Conkling, M. A. (1994). Root-knot nematodedirected expression of a plant root-specific gene. *Science* **263,** 221–223.

Piotte, C., Arthaud, L., Abad, P., and Rosso, M.-N. (1999). Molecular cloning of an acetylcholinesterase gene from the plant parasitic nematodes, *Meloidogyne incognita and Meloidogyne javanica. Mol. Biochem. Parasitol.* **99,** 247–256.

Pritchard, D. I., Brown, A., and Toutant, J.-P. (1994). The molecular forms of acetylcholinesterase from *Necator americanus* (Nematoda), a hookworm parasite of the human intestine. *Eur. J. Biochem.* **219,** 317–323.

Pritchard, D. I., Jagger, C., Green, C., and Lowe, K. (1998). Why do some parasitic nematodes secrete acetylcholinesterase (AChE)? *In* "Structure and Function of Cholinesterases and Related Proteins" (B.P. Doctor *et al.,* Eds.), pp. 509–513. Plenum Press, New York.

Pritchard, D. I., Leggett, K. V., Rogan, M. T., McKean, P. G., and Brown, A. (1991). *Necator americanus* secretory acetylcholinesterase and its purification from excretory-secretory products by affinity chromatography. *Parasite Immunol.* **13,** 187–199.

Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T., and Taylor, P. (1986). Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence. *Nature* **319,** 407–409.

Selkirk, M. E., Hussein, A. S., Russell, W. S., Grigg, M. E., Chacon, M. R., Smith, A. M., Henson, S., and Tippins, J. R. (1998). Secretory acetylcholinesterases from the nematode parasite *Nippostrongylus brasiliensis*. *In* "Structure and Function of Cholinesterases and Related Proteins" (B. P. Doctor *et al.,* Eds.), pp. 515–522. Plenum Press, New York.

Sikorav, J.-L., Duval, N., Anselmet, A., Bon, S., Krejci, E., Legay, C., Osterlund, M., Reimund, B., and Massoulié, J. (1988). Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ. Primary structure of the precursor of the glycolipid-anchored dimeric form. *EMBO J.* **7,** 2983–2993.

Simon, S., Krejci, E., and Massoulié, J. (1998). A four-to-one association between peptide motifs: Four C-terminal domains from cholinesterase assemble with one proline-rich attachment domain (PRAD) in the secretory pathway. *EMBO J.* **17,** 6178–6187.

Stern, B. D. (1986). "Acetylcholinesterase from *Caenorhabditis elegans:* Partial Purification and

Immunocytochemistry of Class C, and Discovery of Class D." Ph.D. thesis dissertation, University of Pittsburg.

Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991). Atomic structure of acetylcholinesterase from *Torpedo californica:* A prototypic acetylcholine-binding protein. *Science* **253,** 872–879.

Sutherland, D., McLellan, J. S., Milner, D., Soong, W., Axon, N., Sanders, M., Hester, A., Kao, Y. H., Poczatek, T., Routt, S., and Pezzementi, L. (1997). Two cholinesterase activities and genes are present in Amphioxus. *J. Expl. Zool.* **277,** 213–229.

Talesa, V., Culetto, E., Schirru, N., Bernardi, H., Fedon, Y., Toutant, J.-P., and Arpagaus, M. (1995). Characterization of a null mutation in *ace-1,* the gene encoding acetylcholinesterase of class A in the nematode *Caenorhabditis elegans. FEBS Lett.* **357,** 265–268.

Talesa, V., Grauso, M., Arpagaus, M., Giovannini, E., Romani, R., and G. Rosi (1999). Molecular cloning and expression of a full-length cDNA encoding acetylcholinesterase in optic lobes of the squid *Loligo opalescens:* A new member of the cholinesterase family resistant to diisopropyl fluorophosphate. *J. Neurochem.* **72,** 1250–1258.

Talesa, V., Romani, R., Grauso, M., Rosi, G., and E. Giovannini (1997). Expression of a single dimeric membrane-bound acetylcholinesterase in *Parascaris equorum. Parasitology* **115,** 653–660.

Taylor, P., and Radic, Z. (1994). The cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol.* **34,** 281–320.

Toutant, J.-P. (1989). Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. *Prog. Neurobiol.* **32,** 423–446.

Toutant, J.-P., Massoulié, J., and Bon, S (1985). Polymorphism of pseudocholinesterase in *Torpedo marmorata* tissues: Comparative study of the catalytic and molecular properties of this enzyme with acetylcholinesterase. *J. Neurochem.* **44,** 580–592.

Vellom, D. C., Radic, Z., Li, Y., Pickering, N. A., Camp, S., and Taylor, P. (1993). Aminoacid residues controlling acetylcholinesterase and butyrylcholinesterase specificity. *Biochemistry* **32,** 12–17.

Waterston, R. H., Sulston, J. E., and Coulson, A. R. (1997). The genome. *In "C. elegans II"* (D. L. Riddle, T. Blumenthal, B. J. Meyer, J. R. Priess, Eds.), pp. 23–45. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

White, J. G., Southgate, E., Thompson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode *C. elegans. Phil. Trans. R. Soc. London. B.* **314,** 1–340.

Xue, D., Finney, M., Ruvkun, G., and Chalfie, M. (1992). Regulation of the *mec-3* gene by the *C. elegans* homeoproteins UNC-86 and MEC-3. *EMBO J.* **11,** 4969–4979.

Zorio, D. A. R., Cheng, N. N., Blumenthal, T., and Spieth, J. (1994). Operons as a common form of chromosomal organization in *C. elegans. Nature* **372,** 270–272.

This Page Intentionally Left Blank

# Reciprocal Regulation of Endothelin-1 and Nitric Oxide: Relevance in the Physiology and Pathology of the Cardiovascular System

Gian Paolo Rossi,[1] Teresa Maria Seccia,[1] and Gastone G. Nussdorfer[2]
[1]Department of Clinical and Experimental Medicine, University Hospital, and
[2]Department of Human Anatomy and Physiology, University of Padua
I-35121 Padova, Italy

The endothelium plays a crucial role in the regulation of cardiovascular structure and function by releasing several mediators in response to biochemical and physical stimuli. These mediators are grouped into two classes: (1) endothelium-derived constricting factors (EDCFs) and (2) endothelium-derived relaxing factors (EDRFs), the roles of which are considered to be detrimental and beneficial, respectively. Endothelin-1 (ET-1) and nitric oxide (NO) are the prototypes of EDCFs and EDRFs, respectively, and their effects on the cardiovascular system have been studied in depth. Numerous conditions characterized by an impaired availability of NO have been found to be associated with enhanced synthesis of ET-1, and vice versa, thereby suggesting that these two factors have a reciprocal regulation. Experimental studies have provided evidence that ET-1 may exert a bidirectional effect by either enhancing NO production via $ET_B$ receptors located in endothelial cells or blunting it via $ET_A$ receptors prevalently located in the vascular smooth muscle cells. Conversely, NO was found to inhibit ET-1 synthesis in different cell types. *In vitro* and *in vivo* studies have started to unravel the molecular mechanisms involved in this complex interaction. It has been clarified that several factors affect in opposite directions the transcription of preproET-1 and NO-synthase genes, nuclear factor-$\kappa$B and peroxisome proliferator-activated receptors playing a key role in these regulatory mechanisms. ET-1 and NO interplay seems to have a great relevance in the physiological regulation of vascular tone and blood pressure, as well as in vascular remodeling. Moreover, an imbalance between ET-1 and NO systems may underly the mechanisms involved in the pathogenesis of systemic and pulmonary hypertension and atherosclerosis.

**KEY WORDS:** Endothelin-1, Nitric oxide, Cardiovascular system, Blood pressure, Hypertension, Atherosclerosis. 

## I. Introduction

A large mass of evidence has been accumulated in the past two decades indicating that endothelial cells play a pivotal role in the control of cardiovascular structure and function (Lüscher and Barton, 1997). The endothelium, acting as an interface located between the vascular wall and the bloodstream, can integrate biochemical and physical stimuli and generate mediators able to affect adjacent vascular smooth-muscle cells (SMCs). These mediators are grouped into two classes: (1) the endothelium-derived relaxing factors (EDRFs), which include nitric oxide (NO), prostacyclin, adrenomedullin (ADM), atrial natriuretic peptide (ANP), and the endothelium-derived hyperpolarizing factor (Brandes *et al.,* 2000), and (2) the endothelium-derived constricting factors (EDCFs), which entail endothelin-1 (ET-1) and thromboxane-$A_2$. In general, EDRFs are considered to play a beneficial role because they enhance natriuresis, inhibit vascular-cell growth and promote apoptosis. In contrast, EDCFs are regarded as playing a detrimental role since they are endowed with activities that can be relevant for cardiovascular damage, such as stimulation of monocyte adhesion, vascular-cell growth and mitogenesis, and inhibition of apoptosis.

Among EDRFs and EDCFs, NO and ET-1 have actracted considerable interest in recent years and have been the object of several review articles (Rubanyi and Botelho, 1991; Bredt and Snyder, 1994; Morris and Billiar, 1994; Rubanyi and Polokoff, 1994; Gray and Webb, 1996; Vanhoutte, 1996; Hocher *et al.,* 1997; Nussdorfer *et al.,* 1997, 1999; Burnstock, 1999; Goldie, 1999; Ignarro, 1999; Jeremy *et al.,* 1999; Kirchengast and Münter, 1999; Miyauchi and Masaki, 1999; Mombouli and Vanhoutte, 1999; Rubino *et al.,* 1999; Russell and Davenport, 1999; Warner, 1999; Li and Föstermann, 2000; Russell and Molenaar, 2000). However, only two of these articles focused on ET-1 and NO interactions: one was restricted to the pulmonary circulation (Rubino *et al.,* 1999), and the other did not survey molecular mechanisms of these interactions (Warner, 1999).

The aim of this article is to review the interplay between ET-1 and NO, with special emphasis on molecular mechanisms involved in the regulation of the prepro(pp)ET-1 gene and endothelial NO synthase (NOS) gene and activity, and on their relevance in the pathogenesis of cardiovascular diseases. Obviously, we will provide an oversimplification of the picture occurring *in vivo,* where many additional factors, each one potentially able to affect ET-1 and NO synthesis and thereby cardiovascular structure and function, are likely to play important roles.

## II. Endothelin-1 and Nitric Oxide Biology

### A. Endothelin-1 Synthesis and Receptors

#### 1. Endothelin-1 Synthesis

ET-1 is a 21-amino-acid peptide (ET-1[1–21]), that is generated from bigET-1 through the action of a specific ET-1 converting enzyme, called ECE-1, which cleaves the precursor molecule at the $Trp^{21}$–$Val/Ile^{22}$ bond (for references, see Rubanyi and Polokoff, 1994; Nussdorfer *et al.,* 1999). More recently, evidence has been provided that bigET-1 may be also cleaved by a chymase, which in humans efficiently converts angiotensin (ANG)-I to ANG-II by splitting the $Phe^{8}$–$His^{9}$ bond (Urata *et al.,* 1990). This chymase cleaves the bigET-1 molecule at the $Tyr^{31}$–$Gly^{32}$ bond, thereby generating ET-1[1–31] (Sanker *et al.,* 1997; Jin *et al.,* 1998), without any further degradation (Nakano *et al.,* 1997). Moreover, the cleavage of big ET-1 by matrix metalloproteinase-2 at $Gly^{32}$–$Leu^{33}$ bond can generate ET-1[1–32] (Fernandez-Patron *et al.,* 1999) (Fig. 1).

#### 2. Endothelin-1 Receptors and Function

The most studied isopeptide of the ET family is the classical ET-1[1–21] (hereinafter ET-1), which acts through two main receptor subtypes: the $ET_A$ receptor located mainly in vascular SMCs and the $ET_B$ receptor found mainly in endothelial cells (Rubanyi and Polokoff, 1994; Miyauchi and Masaki, 1999).

In general, ET-1, through the activation of $ET_A$ receptors, exerts on the cardiovascular system effects opposite those of NO. In fact, besides causing vasoconstriction and thereby raising systemic vascular resistances and blood pressure (Yanagisawa

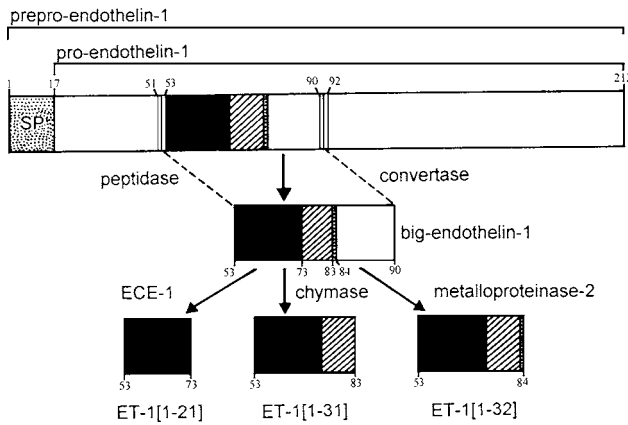



FIG. 1 Biosynthesis and processing of ET-1 from ppET-1. SP, signal peptide.

*et al.,* 1988; Saijonmaa *et al.,* 1991; and for references, see Rubanyi and Polokoff, 1994), ET-1 enhances DNA synthesis and mitogenic activity of vascular cells (Battistini *et al.,* 1993; Kennedy *et al.,* 1993). Hence, ET-1 is deemed to play a crucial role in most conditions characterized by excess vasocontriction and growth of the vascular wall. ET-1[1–31] mimics all the cardiovascular effect of ET-1 (Kishi *et al.,* 1998; Yoshizumi *et al.,* 1998a,b; Inui *et al.,* 1999), thereby being considered to be an agonist of $ET_A$ receptor. ET-1[1–32] has been reported to exert a vasoconstrictor action (Fernandez-Patron *et al.,* 1999).

ET-1 also stimulates aldosterone secretion via the $ET_B$ receptor in the rat (Belloni *et al.,* 1996) and via both $ET_A$ and $ET_B$ receptors in humans (Rossi *et al.,* 1997a,b), an effect that can be relevant for causing extracellular matrix and collagen deposition and therefore fibrosis of the cardiovascular tissue (Weber *et al.,* 1992; Rocha *et al.,* 2000 and for review, see Young and Funder, 2000). Of further interest, ET-1 exerts a strong growth-promoting effect on adrenal zona glomerulosa, acting through $ET_A$ receptors coupled with protein kinase (PK)-C and tyrosine kinase signaling pathways (Mazzocchi *et al.,* 1997, 2001). This effect could be of utmost importance in the regulation of cell turnover of normal adrenal cortex (Nussdorfer *et al.,* 1999; Rossi *et al.,* 2000a).

## B. Nitric Oxide Synthesis and Function

### 1. Nitric Oxide Synthesis

NO is a radical produced from L-arginine (L-Arg) in tissues through the action of NOS. Three different NOSs have been identified: the neural (nNOS, NOS I) and the $Ca^{2+}$-regulated endothelial form (eNOS, NOS III), which are deemed to be constitutively expressed, and the inducible form (iNOS, NOS II), which is expressed in cells upon immunological and inflammatory stimulation (Marletta *et al.,* 1998; Teichert *et al.,* 1998; Andrew and Mayer, 1999; Cosentino and Lüscher, 1999). The most widely investigated in relation to cardiovascular physiology and pathology is the eNOS, and therefore this review will be mostly focused on this isoform.

eNOS functions as a dimer consisting of two identical monomers, which can be functionally divided into two major domains: a C-terminal reductase and a N-terminal oxygenase domain (Fig. 2). Binding sites for one molecule of NADPH, FAD, and FMN are present in the reductase domain, while the oxigenase domain is provided with binding sites for heme, tetrahydrobiopterin ($BH_4$) and the substrate L-Arg. Calmodulin binds to a site stradding the two domains, thus being in a key position to regulate eNOS. The reductase domain is able to transfer electrons from NADPH to cytochrome c via the flavins FAD and FMN. The oxigenase domain transfers electrons to the heme moiety to reduce oxygen, which is used to oxidize L-Arg to NO and L-citrullin (Fig. 2). Although the two domains can function independently under certain circumstances *in vitro,* NOS activity is displayed only in the presence of $Ca^{2+}$/calmodulin and L-Arg. Although eNOS was initially considered to be constitutively expressed, evidence gathered in recent years

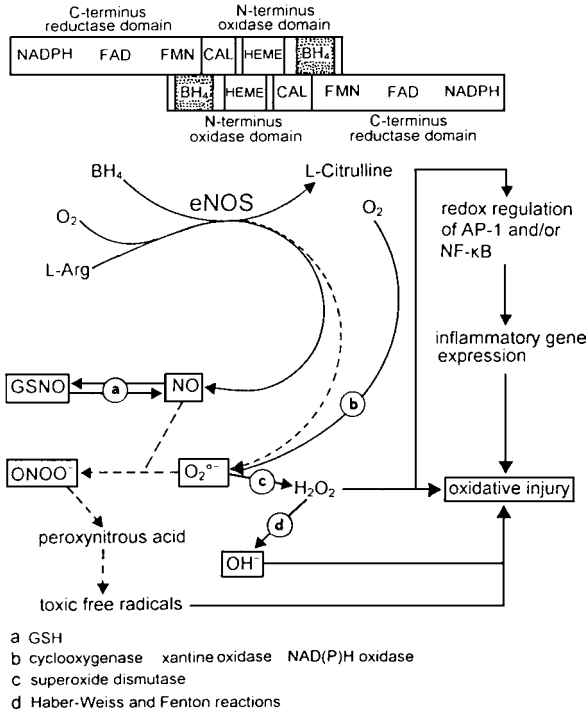



FIG. 2 Schematic representation of eNOS and its bifaceted functional role. In the presence of $O_2^{\circ -}$ and $Ca^{2+}$/calmodulin (CAL) binding to the homodimers, eNOS catalyzes oxidation of L-Arg to L-citrulline with concomitant production of NO. Adequate levels of $BH_4$ are required to activate eNOS. In the presence of low levels of $BH_4$, eNOS leads to generation of $O_2^{\circ -}$ that acts as a NO scavenger, by reacting with NO to generate peroxynitrite ($ONOO^-$). Under physiological conditions, superoxide dismutase converts $O_2^{\circ -}$ to $H_2O_2$, thus preventing $ONOO^-$ production. Furthermore, since $H_2O_2$ is a strong oxidant it can, directly or indirectly via $OH^-$ generated through Haber–Weiss and Fenton reactions, induce oxidative injury. This deleterious effect, which can also be produced by peroxynitrous acid deriving from $ONOO^-$ or other toxic free radicals, is likely to be mediated by activation in endothelial cells and vascular SMCs of redox-sensitive genes, such as those of VCAM-1, ICAM-1, MCP-1, E-selectin, TNF$\alpha$, IL-1, and colony stimulating factor (CSF). *S*-Nitroglutathione (GSNO), derived from NO and glutathione (GSH), is considered an intracellular storage form of NO with putative anti-proatherogenic and anti-proinflammatory effects. Dashed lines indicate reactions activated in presence of low levels of $BH_4$. The dimeric structure and cofactors of eNOS is shown. Each monomer of the two dimers consists of a C-terminal reductase domain containing binding sites for NADPH, FMN, FAD, and CAL and an N-terminal oxygenase domain containing the binding sites for heme and $BH_4$. The two domains exert different functional effects: the reductase domain functions as a cytochrome allowing electrons to be transferred from NADPH, via FAD and FMN, to heme located onto the other subunit, whereas the oxidase domain allows L-Arg to be converted to L-citrulline and NO. Abbreviations as indicated in the text.

(Föstermann *et al.,* 1998) has challenged this view by showing that it undergoes expressional regulation through multiple regulatory steps (see Section III.A).

### 2. Nitric Oxide Function

Following its synthesis in endothelial cells, NO reaches the extracellular space by passive diffusion through the plasma membrane. The principal physiological actions of NO are associated with the activation of the soluble (cytosolic) guanylate cyclase (GC) leading to cyclic-GMP (cGMP) production. cGMP, acting as a second messenger, induces vasodilation in most vascular beds (including brain, coronary, and kidney beds), thereby increasing flow and decreasing vascular resistances and blood pressure (for review, see Ignarro, 1999; Li and Föstermann, 2000).

Recent findings, however, indicate that eNOS is a bifaceted enzyme, which is capable of generating not only NO, but also reactive oxygen species (ROS) (Wever *et al.,* 1998). Under normal conditions and in the presence of adequate amounts of $BH_4$ and cofactors, eNOS synthesizes low concentrations of NO and peroxynitrite, which act as vasodilators and exert an antiatherogenic action. In the presence of low intracellular concentrations of $BH_4$ and/or hyperlipidemia and other risk factors for atherosclerosis, eNOS activity can lead to generation of high amounts of superoxide anion ($O_2^{\circ -}$) and peroxynitrite and thereby can exert toxic effects on the cardiovascular system and promote atherogenesis (for review, see Tomassian *et al.,* 2000; Patel *et al.,* 2000).

*In vivo* studies carried out in a mouse model of eNOS knockout have provided unequivocal evidence that NO, besides contributing to maintain a normal blood pressure, exerts a cell-growth inhibitory effect and is involved in vascular remodeling (Shesely *et al.,* 1996; Rudic *et al.,* 1998; Sato *et al.,* 2000). Part of this effect might be dependent upon the fact that NO interacts with the ET-1 system in the control of angiogenesis and apoptosis. However, there is evidence that NO directly inhibits the proliferation of vascular SMCs, prevents monocyte and leukocyte adhesion to the endothelium and platelet aggregation, and decreases the endothelium permeability to macromolecules and lipoproteins (Wever *et al.,* 1998). Some of these effects may occur through blunting of nuclear factors $\kappa$B(NF-$\kappa$B) (see Section III.C).

## III. Mechanisms of Reciprocal Regulation of Endothelin-1 and Nitric Oxide

### A. Reciprocal Direct Effect

The possibility that there is a direct effect of NO on ET-1 synthesis and vice versa is supported by several lines of evidence. Both the inhibition of NO generation with selective NOS inhibitors and the blockade of NO effects with the soluble GC inhibitor methylene blue lead to increased ET-1 release from porcine aorta *in vitro,* suggesting a direct tonic inhibitory effect of endogenously produced NO on ET-1

synthesis (Boulanger and Lüscher, 1990). In keeping with these findings, it has been demonstrated that the NO donor (FK-409) concentration-dependently decreases ET-1 release from *in vitro*-cultured endothelial cells of both normotensive and DOCA-salt hypertensive rats (Takada *et al.,* 1996). Furthermore, another NO donor (CAS-754) was found to specifically block ET-1-induced, but not ANG-II-induced, vascular SMC proliferation and collagen type-I synthesis (without affecting collagen type-III synthesis), thereby suggesting a modulatory action of NO on ET-1-mediated cardiovascular fibrosis (Rizvi and Myers, 1997).

It is widely documented that ET-1 stimulates NO release from endothelial cells, through $ET_B$ receptors coupled with eNOS (Namiki *et al.,* 1992; Tsukahara *et al.,* 1994; and for references, see Rubanyi and Polokoff, 1994), and the same occurs in mesangial cells (Owada *et al.,* 1994; Beck *et al.,* 1995). Interestingly, the $ET_B$ receptor-mediated eNOS stimulatory effect is more intense in growing than quiescent cells, suggesting a differential expression pattern of endothelin receptor subtypes during the different phases of the cell cycle.

Less known is that ET-1 can also blunt NO release elicited by interleukin (IL)-1$\beta$ from rat vascular SMCs (Ikeda *et al.,* 1997) and cultured mesangial cells (Beck *et al.,* 1995, 1996; Hirahashi *et al.,* 1996). This effect is likely to be mediated by the $ET_A$ receptor, and in vascular SMCs seems to involve the PKC pathway. Since iNOS expression can be induced in most nucleated cells of the heart and vessels (vascular endothelial and endocardial cells, vascular SMCs and cardiac myocytes) (Kanno *et al.,* 1994; Balligard *et al.,* 1995; Iwashina *et al.,* 1996; Papapetropoulos *et al.,* 1999; Pacheco *et al.,* 2000), mechanisms underlying iNOS transcription, in addition to those regarding eNOS, could be of interest. The $ET_A$ receptor-mediated inhibition of cytokine-stimulated NO production by mesangial cells seems to involve suppression of transcription of iNOS and guanosine triphosphate cyclohydroxylase I. The latter is the rate-limiting enzyme of $BH_4$ synthesis (Hirahashi *et al.,* 1996), thus suggesting a very complex interrelationship among ET-1, NO, and NOS cofactors. Consistent evidence, showing iNOS expression in rat carotid arteries after balloon injury (Joly *et al.,* 1992) and in human autherosclerotic lesions (Buttery *et al.,* 1996), also suggests an important role of iNOS at the early stages of atherosclerosis.

Therefore, collectively the herein reviewed findings indicate that, while NO blunts ET-1 synthesis and release, ET-1 can exert a bidirectional effect on NO depending on the cell type, by enhancing its release from endothelium, via $ET_B$ receptors, and decreasing its release from vascular SMCs and other cell types, via $ET_A$ receptors.

## B. Mechanisms Affecting Gene Transcription, Transcript Stability, and Posttranslational Steps

It is conceivable that cardiovascular risk factors and disease mediators act on the regulatory regions of the ppET-1 and NOS genes to elicit changes of their

transcription in opposite directions. In keeping with this contention, molecular investigations of the regulatory regions of these two genes have identified several consensus sequences for binding of regulatory factors. A mammalian transcription-factor activator protein-1 (AP-1) site was recognized in the promoter region of both genes. Factors activating ppET-1 and eNOS gene transcription were found to induce phosphorylation of Jun and Fos proteins, via the PKC cascade, leading to the active form of the AP-1 complex and thus enhancing the AP-1 binding to specific motif of the two genes. Hence, AP-1 may be considered a candidate as common mediator in the transduction process. Cloning of ppET-1 and eNOS genes revealed a variety of *cis* elements for the putative binding of transcription factors, but the signaling cascades triggered by the activators of these transcription factors have been elucidated only in part (Marsden *et al.,* 1993; Wang *et al.,* 1995; Fadel *et al.,* 2000).

Transcription of the ppET-1 gene can be activated by several factors (Fig. 3A), among which are laminar shear stress, hypoxia, ANG-II via AT1 receptors, catecholamines, vasopressin, thrombin, insulin, transforming growth factor-$\beta$ (TGF$\beta$), and low-density lipoprotein (LDL) (Ohta *et al.,* 1990; Emori *et al.,* 1991, 1992; Imai *et al.,* 1992; Gray and Webb, 1996; Kaburagi *et al.,* 1999; Gan *et al.,* 2000; Minchenko and Caro, 2000; Morawietz *et al.,* 2000). The transcription of eNOS gene can be turned on by laminar shear stress, cyclic strain, hypoxia, estrogens, low levels of LDL, $H_2O_2$, ANP and brain natriuretic peptides (BNP), ADM, basic fibroblast growth factor (bFGF), insulin, vascular endothelial growth factor (VEGF), TGF$\beta$, and lysophosphatidylcholine (LPC) (Fig. 3B) (Fleming and Busse, 1999; Samson, 1999; Bilsel *et al.,* 2000; Drummond *et al.,* 2000; Gan *et al.,* 2000; Hinson *et al.,* 2000; Li and Föstermann, 2000). LPC, a major component of oxidized LDL (oxLDL) and perhaps the only activator of eNOS gene expression at present known, was found to increase the binding of Sp-1 to the eNOS promoter. However, oxLDLs may have a bidirectional effect resulting into either suppression or induction of eNOS transcription depending on their concentrations, with low and high concentrations up- and downregulating eNOS mRNA in bovine aortic endothelial cells. A specific TGF$\beta$-responsive element, a partial estrogen responsive element (ERE), and a cyclic-AMP (cAMP)-responsive element (CRE) were also indentified. The other previously mentioned factors might exert their effect by binding to other sites of the eNOS gene, which may stimulate transcription, such as sterol regulatory element (SRE), AP-1, AP-2, and CRE (Papapetropoulos *et al.,* 1999). Of interest, among several polymorphisms in the eNOS gene a $T^{-786}\rightarrow C$ mutation in the 5′-flanking region has been recently identified and found to be associated with coronary spasm in humans and a 52% reduction in the promoter activity (Nakayama *et al.,* 1999). The question of whether this mutation is associated *in vivo* with a blunted NO generation and with an increased plasma and/or tissue levels of ET-1 remains to be investigated.

At present, no information is available on the factors that can affect stability of ppET-1 mRNA. In contrast, evidence has been provided that NO availability can

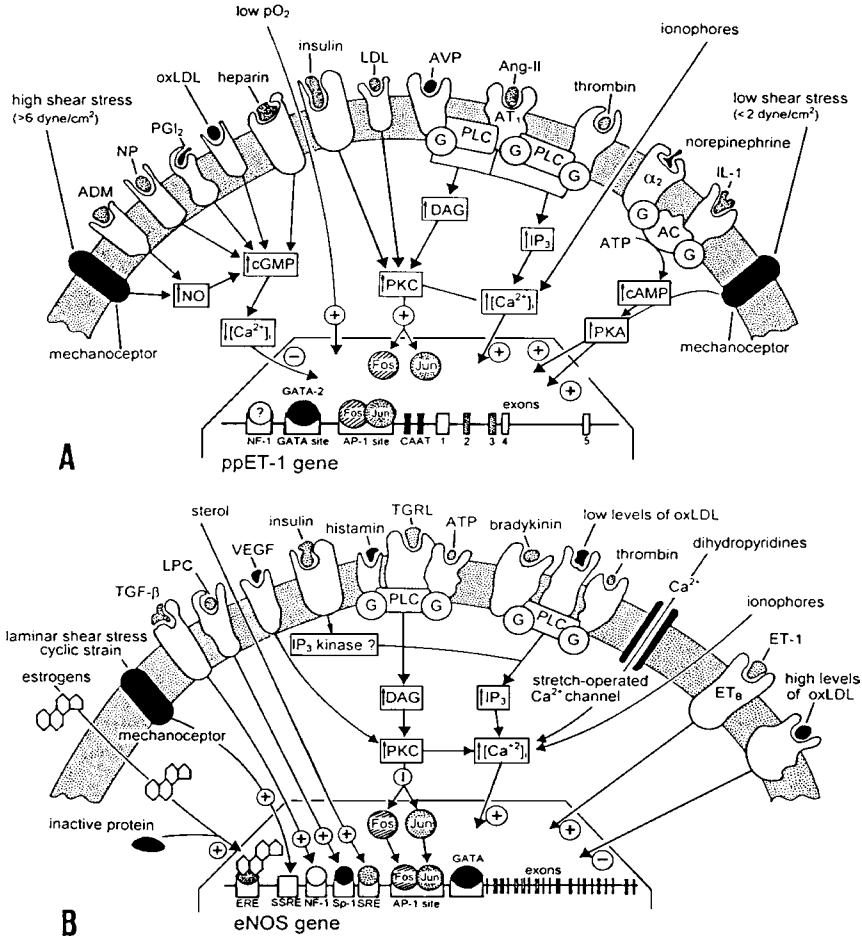



FIG. 3 Schematic representation of the identified factors affecting ET-1 gene (A) and eNOS gene transcription (B) and of the related intracellular signaling pathways. (A) Factors acting via the activation of the phospholipase C (PLC)–PKC pathway, such as insulin, LDL, arginin–vasopressin (AVP), ANG-II, and thrombin, enhance ET-1 production by binding of the AP-1 complex (consisting of a heterodimer Fos–Jun) to the specific motif of the ppET-1-gene promoter. Shear stress also affects, albeit ambivalently, endothelial-cell release of ET-1. Natriuretic peptides (NP), prostacyclin ($PGI_2$), oxLDLs, and heparin blunt ppET-1-gene transcription by increasing cGMP, whereas ADM acts upstream by increasing NO production. Norepinephrine- and IL-1-induced transcription is mediated by the adenylate cyclase (AC)–PKA cascade. In the nucleus a schematic representation of ppET-1 gene depicts exons and consensus sequences in the promoter. (B) Factors acting via activation of the PLC–PKC pathway, such as insulin, histamin, triglyceride-rich lipoproteins (TGRL), ATP, bradykinin, low levels of oxLDLs, and thrombin, enhance the synthesis of NO by binding of the AP-1 complex to the specific site in the eNOS-gene promoter. Interestingly, oxLDLs have a bidirectional effect, depending on their intracellular levels: low levels stimulate and high levels inhibit eNOS expression. ET-1 can also increase NO release via $ET_B$ receptors located in endothelial cells. Since eNOS is a $Ca^{2+}$-dependent enzyme, $Ca^{2+}$ ionophores enhance eNOS gene transcription. Some consensus sequences for transcription factors and the corresponding factors are schematically reproduced in the promoter: ERE, shear stress responsive element (SSRE), nuclear factor-1 (NF-1), serum response element, AP-1 and GATA sites. DAG, diacylglycerol; G, G proteins; $IP_3$, inositol triphosphate. Other abbreviations as indicated in the text.

be regulated by factors affecting stability of eNOS gene transcripts: tumor necrosis factor-$\alpha$ (TNF$\alpha$), hypoxia, and oxLDLs decrease it, and inhibitors of HMG-CoA reductase, $H_2O_2$, and VEGF increase it (Alonso *et al.,* 1997; Papapetropoulos *et al.,* 1999; Drummond *et al.,* 2000).

Again no findings are available enlighting the possibility that ET-1 can be regulated at posttranslational steps, while investigations suggested that this occur for NOS activity. Studies demonstrating heterocomplex assembly of the NOS isoforms with a variety of regulatory and structural proteins clearly have added a further level of complexity to NOS regulation by indicating the possibility of protein–protein interactions (Kone, 2000). Evidence of posttranslational modification (phosphorylation, myristilation, and palmytoilation) and intracellular translocation (membrane bound versus cytoplasm and interaction with *caveolae*) is also available and strongly supports the concept that eNOS activity is tightly regulated by environmental factors (Föstermann *et al.,* 1998; Bloch, 1999; Drummond *et al.,* 2000; Kone, 2000).

## C. Mechanisms Acting through a Common Mediator

Cardiovascular risk factors can act through a common mediator, which affects in opposite directions ppET-1 and eNOS genes. A likely candidate as common mediator could be $O_2^{\circ -}$, which is known to stimulate NF-$\kappa$B formation (Fig. 2).

It has been recently shown that availability and biological effects of NO are modulated by the local concentration of $O_2^{\circ -}$ in tissues and that the balance between these two radicals can be far more important for cardiovascular pathophysiology than the absolute levels of either alone (McIntyre *et al.,* 1999). Indeed, $O_2^{\circ -}$ can act as a scavenger by reacting with NO, causing its breakdown and leading to peroxynitrite formation (Fig. 2) (McIntyre *et al.,* 1996). However, $O_2^{\circ -}$ can also affect NO availability by modulating eNOS-gene expression either directly or indirectly by generating other ROS.

$H_2O_2$ has been reported to increase eNOS-gene expression in bovine and human aortic endothelial cells by raising both the rate of transcription and the stability of its transcript (Drummond *et al.,* 2000). This findings is consistent with the demonstration of the presence of binding sites for redox-sensitive transcription factors, such as AP-1, Sp-1 and antioxidant-responsive elements in human, bovine, and murine eNOS-gene promoter regions (Robinson *et al.,* 1994; Venema *et al.,* 1994; Teichert *et al.,* 1998). Additional data also strengthen the contention that ROS play a crucial role in the regulation of both NO and ET-1 availability. The cyclosporine-induced transcription of eNOS gene in bovine aortic endothelial cells was found to be mediated by ROS, which activate AP-1 allowing its binding to the responsive *cis*-regulatory sequence located in the 5′-regulatory region. Since both catalase and superoxide dismutase abolished this effect of cyclosporine, it is likely that $O_2^{\circ -}$ and $H_2O_2$ are mediators of this pathway (Lopez-Ongil *et al.,*

1998; Navarro-Antolin *et al.,* 1998). Endogenously or exogenously derived ROS can affect ET-1 production, at least in mesangial cells, because $H_2O_2$ was found to increase and $O_2^{\circ -}$ to lower it (Hughes *et al.,* 1996). Superoxide has been also reported to modulate ET-1 production in vascular endothelial cells by regulating ECE-1 activity (Lopez-Ongil *et al.,* 2000). Since consensus sequences for AP-1 are present in both ppET-1 and eNOS genes and ROS modulate both ET-1 and NO synthesis, it is likely that ROS act as common mediators of expression of these two regulatory molecules. This contention is also supported by evidence of enhanced AP-1 DNA binding activity in endothelial cells and vascular SMCs after exposure to $H_2O_2$ and oxLDLs.

NO itself is a ROS, and therefore it can modulate gene expression by altering the intracellular oxidative environment. The final effect may be either beneficial or detrimental, depending on the cell type and the prevailing intracellular NO level. NO can reduce superoxide generation and inhibit LDL oxidation, as well as produce the strong oxidant peroxynitrite anion. Interestingly, intracellular ROS formation can also be controlled by ET-1, suggesting a further possibility of reciprocal interaction between ET-1 and NO. Indeed, it has been recently demonstrated that ET-1 concentration-dependently induces, via $ET_B$ receptors, the expression of gp9Iphox, the limiting subunit of the forming NAD(P)H oxidase, in cultured endothelial cells of human umbelical vein, thus raising $O_2^{\circ -}$ generation (Duerrschmidt *et al.,* 2000). The increased $O_2^{\circ -}$ levels could in turn enhance peroxynitrite formation and/or induce oxidative changes in LDL, leading to the same final harmful effect, i.e., a reduced NO bioavailability. However, the possibility that ET-1 increases the oxidative stress has been contended by Ishizuka *et al.,* (1999), based on the finding that the activation of $ET_B$ receptors increases NO production and suppresses the expression of vascular cell adhesion molecule-1 (VCAM-1) in human endothelial cells. Whether $ET_B$-receptor stimulation in endothelial cells actually entails a bidirectional effect on NO release is controversial and remains to be fully elucidated. Differences in prevailing redox environment, vascular beds, or cell types might explain these divergent results. In keeping with this contention, other investigators observed that ET-1 raises intracellular ROS levels in cardiac myocytes via the activation of the $ET_A$ receptor (Cheng *et al.,* 1999).

### 1. Role of Nuclear Factors $\kappa$B

NF-$\kappa$B are a family of at least five trascription factors (p50, p52, p65, c-Rel, Rel-B), which act as essential regulators of the expression of several genes involved in immune, inflammatory, and growth responses (Gallois *et al.,* 1998). They form homo- or heterodimers and bind to a DNA sequence, named $\kappa$B motif (Fig. 4). Several known NF-$\kappa$B activators (including cytokines, phorbol esters, growth factors, and viral transactivators) rapidly phosphorylate the inhibitory protein I-$\kappa$B, leading to the release of free NF-$\kappa$B dimers. These latter translocate into

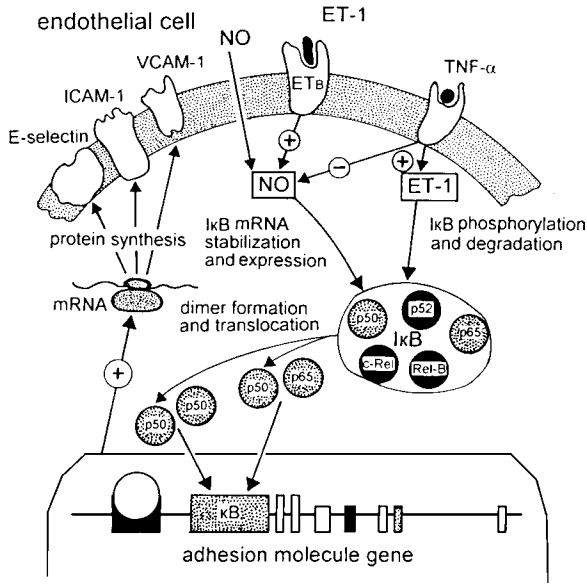



FIG. 4 Proposed model of NF-$\kappa$B as a common mediator in gene transcription activated by ET-1 and NO. Genes coding for pro-atherogenic and pro-inflammatory molecules, such as VCAM-1, ICAM-1, and E-selectin, share specific DNA binding motifs in their promoters for interaction with NF-$\kappa$B. NO inhibits, whereas ET-1 enhances NF-$\kappa$B activation, leading to a blunted or an increased synthesis of pro-atherogenic molecules, respectively. The inhibitory effects of NO are mediated by a tonic inhibition of NF-$\kappa$B, a stabilization of I-$\kappa$B and an increased I-$\kappa$B mRNA expression. In contrast, ET-1 can modulate NF-$\kappa$B activation by promoting I-$\kappa$B phosphorylation and degradation, which in turn lead to a reduced stability of the inactive complex and thus enhance binding of the active complex to the NF-$\kappa$B site. The complex consisting of transcription factors p50, p52, p65, c-Rel-B, plus the inhibitory factor I-$\kappa$B, represents the inactive form. Phosphorylation of I-$\kappa$B$\alpha$ induces destabilization of the complex and subsequent release of the dimeric complex p50/p50 or p50/p65, namely the active form of NF-$\kappa$B, able to bind the specific motif $\kappa$B in the promoter. Abbreviations as indicated in the text.

the nucleus, where they activate gene expression of several molecules involved in atherogenesis, including the adhesion molecules VCAM-1, intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), E-selectin, and the inflammatory cytokines IL-6 and IL-8. These genes share specific $\kappa$B motifs in their promoter regions.

It has been demonstrated that NO inhibits the activation of NF-$\kappa$B through different mechanisms, including stabilization and enhanced expression of I-$\kappa$B (Peng *et al.,* 1995). ET-1 exerts opposite effects because, acting via the $ET_B$ receptor, it was shown to increase the formation of the two NF-$\kappa$B DNA binding complexes, the p50/p50 homodimer and the p50/p65 heterodimer, as well as induce the degradation of the inhibitory protein 1-$\kappa$B-$\alpha$, thus releasing NF- $\kappa$B monomers (Gallois

*et al.,* 1998) (Fig. 4). In turn, overexpression of NF-$\kappa$B p65 was shown to induce ET-1 gene transcription in cultured bovine aortic endothelial cells (Quehenberger *et al.,* 2000).

The finding of activated NF-$\kappa$B in the coronary vessel of hypercholesterolemic pigs, which show decreased NO bioavailability (i.e., an early marker of atherosclerosis), further supports the hypothesis that NF-$\kappa$B activation, driven by an imbalance between ET-1 and NO, might be crucial for the early steps of atherogenesis (Wilson *et al.,* 2000). This contention is also strengthened by the observations that (1) NO exerts a potent inhibitory effect on the transcription of molecules regulating endothelial-cell adhesiveness (e.g., VCAM-1 and MCP-1) through an oxidant-responsive transcriptional pathway and (2) ET-1 stimulates the synthesis of IL-6, through an $ET_A$-mediated pathway linked to NF-$\kappa$B, an effect that can be enhanced by ROS (Browatzki *et al.,* 2000).

## 2. Role of Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) play a pivotal role in lipid and glucose metabolism, and have been implicated in metabolic disorders predisposing to atherosclerosis (for references, see Buchan and Hassall, 2000; Robert-Thomson, 2000). Both PPAR$\alpha$ and PPAR$\gamma$ were found to be expressed in human endothelial cells, as well as in macrophages, while only PPAR$\alpha$ is expressed in SMCs from human aorta (Delerive *et al.,* 1999; Bishop-Bailey, 2000; Ikeda *et al.,* 2000). PPAR$\alpha$ and PPAR$\gamma$ activators were shown to negatively modulate NF-$\kappa$B signaling pathway in vascular SMCs and monocyte-derived macrophages, respectively. Accordingly, they have anti-inflammatory effects that can be relevant in atherosclerosis (Ikeda *et al.,* 2000).

At present, only few investigations dealt with the effects of PPARs on ET-1 and NO synthesis. It has been shown that the PPAR$\gamma$-ligands troglitazone and 15-deoxy-$\Delta^{12,14}$ prostaglandin-J2 inhibit NO synthesis in rat vascular SMCs, blunt the activation of NF-$\kappa$B, and lower iNOS activity stimulated by cytokines (Ikeda *et al.,* 2000). These findings suggest that PPAR$\gamma$-blunted NO synthesis may contribute to the development and progression of the atherosclerotic lesions. The activators of PPAR$\alpha$ and PPAR$\gamma$ fenofibric acid and troglitazone, respectively, were reported to inhibit thrombin-induced ppET-1 gene expression in and ET-1 release from human vascular endothelial cells, by preventing the binding of AP-1 proteins to the specific sequence in the gene promoter. In contrast, these PPAR activators did not affect per se unstimulated ET-1 production, a finding consistent with the lack of putative PPAR-responsive elements in the promoter region of the ppET-1 gene (Delerive *et al.,* 1999). Thus, collectively these results suggest that PPARs play an important role in the functional regulation of endothelial cells and vascular SMCs, possibly acting as common mediators in the transcription of genes involved in the synthesis of ET-1 and NO.

## IV. Physiological and Pathophysiological Relevance of Endothelin-1 and Nitric Oxide Interplay

Given the opposite effects of ET-1 and NO on the cardiovascular system, it is likely that these two regulatory molecules play a relevant role in the physiological regulation of vascular tone and blood pressure. Moreover, it is reasonable to conceive that an imbalance between ET-1 and NO production may be involved in the pathogenesis of systemic and pulmonary hypertension, congestive heart failure, atherosclerosis, and renal damages, i.e., in diseases where vasoconstriction and/or vascular-cell proliferation are increased. "Endothelial dysfunction," defined as an imbalance between increased ET-1 and decreased NO productions, was found to be associated with most cardiovascular risk factors, such as hyperlipidemia, diabetes mellitus, systemic hypertension and family history of hypertension, cigarette smoking (Miyauchi and Masaki, 1999; Li and Föstermann, 2000), and, more recently, hyperhomocyst(e)inemia (Doshi *et al.,* 1999; Eikelboom *et al.,* 1999). Therefore, endothelial dysfunction could be regarded as an early step or a precursor of atherogenesis, ultimately leading to cardiovascular events. As such, it might represent an important target for pharmacological intervention aimed at preventing cardiovascular diseases.

### A. Regulation of Vascular Tone and Vascular Remodeling

#### 1. Vascular Tone

Recent *in vivo* studies have clearly demonstrated that ET-1 and NO interact in the regulation of vascular tone in humans. The effects of $ET_A$- and $ET_B$-receptor blockade by selective antagonists (BQ-123 and BQ-788, respectively) on the forearm blood flow were investigated in healthy normotensive subjects (Verhaar *et al.,* 1998). In two separate studies, it was found that $ET_A$-receptor blockade causes significant vasodilation, which is reduced by 95% by the concomitant inhibition of endogenous NO production ("NO clamp" with the administration of a NOS inhibitor plus sodium nitroprusside to restore baseline values of forearm blood flow) and by 38% by the concomitant administration of BQ-788. BQ-788 alone caused a 20% decrease in forearm blood flow. It has been concluded that the acute selective $ET_A$-receptor blockade evokes vasodilation of human forearm resistance vessels, which mainly results from enhanced NO generation. At variance, the $ET_B$-receptor blockade, either alone or on a background of $ET_A$-receptor antagonism, causes vasoconstriction, thus indicating that $ET_B$-receptors predominantly mediate ET-1-induced vasodilation. Cardillo *et al.* (2000), using an analogous experimental model, investigated the effects of $ET_A$-, $ET_B$-, or combined $ET_A$- and $ET_B$-receptor blockade. Although any significant vasodilation was observed in response to $ET_A$-receptor blockade, they concluded that ET-1 contributes to the physiological

regulation of vascular tone by stimulating NO production via the $ET_B$ receptors. These investigators also suggested that the vasoconstrictor effect of ET-1 may remain unopposed in conditions of reduced NO activity, such as hypertension and atherosclerosis.

Quite different and sometimes conflicting results were obtained in experimental animals. Ming *et al.* (1998) reported that in the dog coronary artery the $ET_A$-receptor blockade, alone or combined with $ET_B$-receptor antagonism, partially restores the vasodilatory response to acetylcholine after NOS inhibition, the $ET_B$-receptor blockade alone being ineffective. On these grounds, they suggested that the $ET_A$ receptor-dependent rise in vascular tone magnifies the inhibitory effect of NO formation blockade on the acetylcholine-induced vasodilation, at least in the resistance coronary vessels. According to Gellai *et al.* (1996), *in vivo* in the rat the predominant role of endogenous endothelins is an $ET_B$-receptor mediated vasodilation, $ET_A$ receptors playing a negligible role in the control of vascular tone. Opposite results were obtained using a model of *in situ* perfusion of the adrenal gland, where ET-1 causes increase in vascular resistances, the effect of the activation of $ET_A$ receptors overcoming that of $ET_B$ receptors. $ET_A$-receptor blockade and NOS inhibition per se decreased and increased, respectively, adrenal vascular resistance, thereby suggesting that endogenous endothelins and NO exert tonic vasoconstrictor and vasodilatory action, respectively (Hinson *et al.,* 1991, 1996). Mazzocchi *et al.* (1998) did not confirm these last findings, because neither BQ-123 and BQ-788 nor NOS inhibitors were per se able to affect vascular resistances in in situ perfused rat adrenal gland. Moreover, they showed that the inhibition of endogenous NO generation abolishes the $ET_B$ receptor-induced decrease in adrenal vascular resistance and that $ET_B$-receptor blockade magnifies the vasoconstrictor effect of ET-1.

Differences related to species and vascular beds might account for these different results. Nonetheless, collectively available evidence strongly suggests that the interaction between ET-1 and NO is crucial in the control of vascular tone under physiological conditions.

## 2. Vascular Remodeling

Elegant *in vitro* studies provided evidence that ET-1 and NO cooperate in angiogenesis and vascular remodeling. By using Chinese hamster ovary cells transfected with $ET_B$-receptor and/or eNOS cDNAs, Goligorsky *et al.* (1999) showed that both functional $ET_B$ receptors and eNOS activity are needed for endothelial-cell migration in response to ET-1 to occur. Shichiri *et al.* (2000) reported that ET-1 inhibits NO-induced apoptosis in vascular SMCs, acting through $ET_A$ receptors coupled with the mitogen-activated protein kinase (MAPK) pathway. More recently, vascular SMC proliferation, but not migration, was found to be inhibited by apolipoprotein-E through a mechanism involving activation of iNOS (Ishigami *et al.,* 2000). The differential involvement of all

these mechanisms associated with the endothelial-cell biology is likely to reflect multiple regulatory steps, thus pointing again to the complexity of these processes.

## B. Experimental and Human Hypertension and Atherosclerosis

### 1. Experimental Investigations

The acute NOS inhibition by $N^G$-nitro-L-arginine (L-NAME) was found to cause hypertension in rats, which develops over time and can be prevented by the $ET_A/ET_B$-receptor antagonist bosentan (Banting *et al.,* 1996). At variance, long-term L-NAME administration was not counteracted by bosentan (Moreau *et al.,* 1995). However, even despite the lack of any blood-pressure lowering effect, bosentan was able to markedly decrease extracellular-matrix deposition and fibrosis in the kidney glomeruli, clearly indicating a dissociation between the mechanisms involved in raising of blood pressure and those implicated in vascular kidney damage (Tharaux *et al.,* 1999).

Several studies stressed the crucial role of ET-1 and NO interactions in atherogenesis. To address the question on whether ET-1 contributes to atherogenesis and plaque activation through ROS, Fei *et al.* (2000) investigated the effects of ET-1 on ROS and c-Jun amino-terminal kinase (JNK) and extracellular signal regulated kinases (ERK) 1 and 2 in the rat vascular SMCs. They directly demonstrated that ET-1 causes a time-dependent generation of ROS, and also showed that ET-1-induced JNK activation is prevented by both the radical scavenger *N*-acetylcysteine and the inhibitor of NADPH oxidase diphenylene iodonium. In light of these findings, these investigators concluded that ET-1 induces, via NADP oxidase, generation of ROS, which act as second messenger to activate JNK and thus inflammatory responses in atherosclerotic plaques.Ishizuka *et al.* (1999) found that the blockade of endogenous NO production by the NOS inhibitor L-NO-monomethylarginine (L-NMMA) increases the surface expression and mRNA accumulation of the adhesion molecule VCAM-1 in human vascular endothelial cells stimulated with ET-1 and that ET-1 enhances these parameters in cells pretreated with TNF$\alpha$. In contrast, ET-1 alone was ineffective. Since pretreatment with BQ-788 prevented the synergistic enhancement of VCAM-1 expression induced by ET-1 in TNF$\alpha$-stimulated cells, it is conceivable that activation of $ET_B$ receptors in human endothelial cells may result in the production of NO, which in turn suppresses VCAM-1 expression. The inhibitory effect of TNF$\alpha$ on NO production, via destabilization of eNOS mRNA, could explain why ET-1 increases VCAM-1 expression in TNF$\alpha$-pretreated cells, thus supporting the contention of a strict relationship between ET-1 and NO in human endothelial cells at the early stages

of atherosclerosis (Alonso *et al.,* 1997). An involvement of a NF-$\kappa$B-dependent mechanism in this relationship is suggested by the fact that (1) genes encoding for pro-atherogenic and pro-inflammatory molecules (VCAM-1, ICAM-1, MPC-1, E-selectin, IL-6, and IL-8) share specific DNA binding motifs in their promoter regions for interaction with NF-$\kappa$B; and (2) NO inhibits NF-$\kappa$B activation (Fig. 4). Accordingly, Peng *et al.* (1995) demonstrated that NO exerts a tonic inhibition of NF-$\kappa$B in human endothelial cells, probably through the stimulation of I-$\kappa$B expression and the stabilization of I-$\kappa$B mRNA. ET-1 can also modulate NF-$\kappa$B activation, inasmuch as it was found to induce IL-6 release in human vascular SMCs via the activation of NF-$\kappa$B (Browatzki *et al.,* 2000). Taken altogether, these findings strongly support to the possibility that the ET-1/NO cross-talk, mediated by a common NF-$\kappa$B-dependent mechanism driven in opposite way by the two regulatory molecules, could be of utmost importance for triggering the atherogenic process.

These *in vitro* observations are undoubtely of much interest. However, they do not conclusively prove that interactions between ET-1 and NO are of relevance *in vivo* in the pathogenesis of atherosclerosis. To address this issue, Barton *et al.* (1998a) used the ApoE knockout mice, a model of atherogenesis resembling human atherosclerotic disease since these animals develop severe aortic atherosclerotic lesions even without receiving a high-cholesterol diet. Treatment with an $ET_A$ selective antogonist for 30 weeks decreased by about 30–40% aortic atherosclerotic changes, despite leaving unaffected plasma levels of lipids. The extent of this reduction of atherosclerosis was associated with a significant decrease in ET-1 content in the aorta and a normalization of endothelium-dependent relaxation. As these changes were coupled with a marked rise in plasma nitrites, they would indicate a restoration of NO bioavailability during prolonged $ET_A$-receptor blockade. A straightforward approach to the question on whether NO exerts an antiatherogenic effect has been undertaken by supplementing mice, lacking functional LDL receptors and fed a high-cholesterol diet, with L-Arg alone or associated with a NOS inhibitor (Aji *et al.,* 1997). It has been observed that L-Arg significantly reduces aortic atherosclerosis, as compared to both control animals and animals receiving both L-Arg and the NOS inhibitor, thereby providing convincing evidence that L-Arg supplementation exerts an antiatherogenic action by releasing NO. Additional evidence of an important role *in vivo* of an imbalance between ET-1 and NO comes from experiments carried out in pigs made hypercholesterolemic with a high cholesterol diet and chronically administered either a mixed $ET_A/ET_B$- or an $ET_A$-selective antagonist (Best *et al.,* 1999). It was observed that both treatments restore coronary vasodilatory response to bradykinin, attenuate the decrease in plasma NO and coronary-artery eNOS immunoreactivity, and increase hypercholesterolemia-induced rise in the plasma levels of $F_2$-isoprostanes, which is a marker of lipid peroxidation with vasoconstrictory effect. More recently, it has been reported that eNOS immunoreactivity is decreased, while iNOS and

ET-1 immunoreactivities are increased in aortic endothelial cells of rats kept on a cholesterol-enriched diet (Aliev *et al.,* 2000). On these grounds, the conclusion was drawn that chronic $ET_A$ antagonism preserves endothelial function, increases NO production, and decreases oxidative stress, all mechanisms that are relevant for atherogenesis.

## 2. Human Studies

The *in vitro* and *in vivo* experimental findings discussed above raise the question of whether the interactions between ET-1 and NO are relevant also in human diseases. A number of studies undertaken to address this issue have consistently shown a blunted vasodilatory response to acetylcholine in the forearm vascular bed of patients with hypertension (Taddei *et al.,* 1993a,b) and atherosclerosis (Drexler and Zeiher, 1991; Zeiher *et al.,* 1991; Drexler and Hornig, 1999) compared to that seen in healthy control subjects. In human essential hypertension, as well as in secondary forms of hypertension, the vasodilatory response to acetylcholine is decreased, suggesting impaired availability of NO or a blunted cGMP-mediated pathway (Linder *et al.,* 1990; Panza *et al.,* 1990; Taddei *et al.,* 1993a,b; Taddei and Salvetti, 1997). This impaired endothelium-dependent relaxation could be attributed to at least three mechanisms: (1) functional abnormalities in eNOS activity, possibly due to environmental and/or genetic factors (Nakayama *et al.,* 1999); (2) increased NO breakdown; and (3) reduced eNOS expression (Quyyumi *et al.,* 1997). On the other hand, the evidence that the ET-1 system is activated in hypertension and atherosclerosis is to some extent still controversial. Several studies failed to detect significant differences in ET-1 plasma levels between hypertensive patients and normotensive subjects, but most had an inadequate statistical power due to a too small sample size (Rossi *et al.,* 2000b). However, it must be remembered that ET-1 secretion from endothelial cells is polarized (Wagner *et al.,* 1992) and occurs abluminally (i.e., toward the *tunica media*). Thus it is not surprising that ET-1 plasma levels are not markedly elevated. To investigate the possibility that the concentration of ET-1 in the *tunica media* is more meanigful than that in the plasma, an immunocytochemical study was carried out in arteries from young normotensive organ donors and patients with hypertension and coronary artery disease (CAD) (Rossi *et al.,* 1999). It was found that while ET-1 is detectable almost exclusively in the endothelium of normotensive subjects, it is easily found in the *tunica media* of the thoracic artery of hypertensive and CAD patients. Both immunoreactive ET-1 and the mRNAs of ppET-1 and ECE-1 were demonstrated, not only in endothelium- and adventitia-deprived arteries, but also in isolated aortic SMCs. Collectively, these findings indicate that in human beings with hypertension and/or CAD ET-1 synthesis is enhanced and occurs through the enrolment of cell types, as vascular SMCs, which under normal conditions do not synthesize this peptide. However, the question on whether this precedes, concurs, or follows a decrease in NO production, remains

to be investigated. Of further interest, a regression analysis showed that the intensity of ET-1 immunostaining in the *tunica media* correlates directly with the mean blood pressure and the plasma concentration of total cholesterol, and inversely with the number of sites of atherosclerotic lesions (Rossi *et al.,* 1999). These findings, along with the demonstration that (1) there is a direct relation between the plasma levels of ET-1 and the number of atherosclerotic sites (Lerman *et al.,* 1991) and the severity of coronary-artery stenoses (Salomone *et al.,* 1996); (2) ET-1 immunoreactivity and ppET-1 mRNA are present in coronary-artery plaques (Winkles *et al.,* 1993; Zeiher *et al.,* 1993); and (3) coronary atherosclerotic plaques subjected to stretching during ballon dilatation progressively release ET-1 (Hasdai *et al.,* 1997), suggest that plasma ET-1 level can be taken as a marker of enhanced release of ET-1 from arteries with advanced atherosclerosis. They also provide support for a role of this peptide in CAD, spasm during percutaneous transluminal coronary angioplasty (PTCA), and possibly hypertension.

eNOS was investigated by immunocytochemistry in human arteries with and without atherosclerosis (Oemar *et al.,* 1998). eNOS immunostaining was found to be decreased in the latter compared to the former, a finding that cannot be imputed to methodological factors since staining in the endothelium of *vasa vasorum* was well preserved in both types of arteries. To corroborate the contention that NO availability is actually decreased, these authors used a NO-sensitive microelectrode and showed that both the initial rate and the peak release of NO in response to a $Ca^{2+}$ ionophore were markedly decreased in the atherosclerotic carotid as compared to normal arteries. These data are consistent with those obtained in patients with atherosclerotic involvement of the peripheral arteries, which also suggested an impaired NO synthesis (Bode-Boger *et al.,* 1994, 1996a,b). In light of these data, it has been proposed that atherosclerosis could be regarded as an arginine-deficiency disease (Cooke and Tsao, 1997; Cooke, 1998).

In contrast with the wealth of studies dealing with either ET-1 or NO alone, few investigations have been undertaken to investigate both factors at the same time. A blunted forearm blood-flow response to the eNOS inhibitor L-NMMA, associated with an enhanced vasodilatory response to the mixed $ET_A/ET_B$-receptor antagonist TAK-044, was observed in essential hypertensive patients compared to normal subjects (Taddei *et al.,* 1999), a finding suggesting the existence of both impaired tonic NO release and increased vasoconstriction to endogenous ET-1. To further address this problem, patients with CAD undergoing coronary angiography were studied (Lerman *et al.,* 1995; Burnett, 1997). It was shown that patients who exhibit no vasodilation (i.e., an abnormal coronary response to acetylcholine) display an increase in ET-1 and no rise in cGMP, while patients possessing a normal vasodilatory response to acetylcholine have increased cGMP and unchanged ET-1. Unfortunately, no gene expression and immunocytochemical studies, as those surveyed above, have been carried out on both ET-1 and NO systems in the same arteries. Thus, the concept that ET-1 and NO undergo inverse changes in atherosclerosis remains to be conclusively proven.

### C. Pulmonary Hypertension

Primary and secondary pulmonary hypertensions are ominous diseases for which available pharmacological treatments are rather discouraging. There is strong evidence of an imbalance between ET-1 and NO synthesis in both types of pulmonary hypertension (Barer *et al.,* 1993; Giaid *et al.,* 1993, 1995; Dupuis *et al.,* 1998; Hampl and Herget, 2000).

The vasculature of the lung is a site of both intense ET-1 production and clearance, which can be altered in lung diseases and pulmonary hypertension (Dupuis *et al.,* 1998). The $ET_A$ receptors were found to be highly expressed in both conduit and resistance arteries of the lung, and therefore this vascular bed is exquisitely sensitive to the vasoconstrictor effect of ET-1 (Dupuis *et al.,* 1998). ET-1 has also been shown to exert a clear-cut proliferogenic effect on pulmonary artery SMCs via $ET_A$ receptors (Zamora *et al.,* 1993). In addition, the transcription of the ppET-1 gene is turned-on by hypoxia (see section III.B), and several investigations have clearly documented both an activation of the ET-1 system and a beneficial effect of endothelin-receptor antagonists on the course of pulmonary hypertension (Chen and Oparil, 2000). In keeping with the concept of an imbalance between ET-1 and NO in pulmonary hypertensive patients, overexpression of eNOS in the pulmonary vessels was found to ameliorate the course of monocrotaline-induced experimental pulmonary hypertension (Campbell *et al.,* 1999), and a similar effect was obtained with the administration of the NO donor L-Arg (Mehta *et al.,* 1995).

### D. Strategies for Restoring a Normal ET-1/NO Balance

Several endothelin-receptor antagonists have been developed, and some are under investigation in hypertension and congestive heart failure (Kiowski *et al.,* 1995; Krum *et al.,* 1998; Sutsch and Kiowski, 2000). Given the reciprocal regulation of ET-1 and NO, it is likely that blockade of the former would results into improvement of the latter. The findings obtained in ApoE knockout mice and other models, where chronic treatment with the selective $ET_A$-receptor antagonist LU-135252 was found to lower ET-1 content in the arterial wall and to restore NO-mediated vasodilation, are consistent with a potential role of endothelin-receptor antagonists in restoring a normal balance between ET-1 and NO (Barton *et al.,* 1998a,b).

There is overwhelming evidence that the impaired vasodilatory response observed in patients with hypertension and atherosclerosis could be reversed by either administration of supplemental L-Arg or by other interventions aimed at restoring NO availability, such as administration of the eNOS cofactor $BH_4$ (Bode-Boger *et al.,* 1994, 1996a; Hirata *et al.,* 1995; MacAlister *et al.,* 1996; Boger *et al.,* 1998; Setoguchi *et al.,* 1999). These observations appeared at first paradoxical, since the $K_m$ of eNOS for L-Arg is in the picomolar range and the plasma and intracellular concentrations of L-Arg are 100- and 1000-fold higher, respectively. However, they may be explained by the identification of circulating inhibitors of

NOS, such as $N^G$,$N^G$-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) and L-NMMA (Vallance *et al.,* 1992; Bode-Boger *et al.,* 1996b; Matsuoka *et al.,* 1997). ADMA was found to be elevated from 2- to 10-fold under conditions associated with endothelial vasodilator dysfunction, such as hypertension, hypercholesterolemia, and diabetes (Cooke and Tsao, 1997; Cooke, 1998). These plasma levels of ADMA are likely to be sufficient to impair NO-mediated vasodilation (i.e., endothelium-dependent vasodilation).

An alternative or complementary strategy to restore NO-mediated effects could be the prevention of $O_2^{\circ -}$ formation and/or its scavenging. By decreasing NF-$\kappa$B a reduction of $O_2^{\circ -}$ would be expected to enhance the transcription of eNOS and to blunt that of ppET-1 gene (see section III.B). This contention appears to be supported by the demonstration that in double transgenic rats harboring both human renin and angiotensinogen genes, the inhibition of NF-$\kappa$B with pyrrolidine dithiocarbamate markedly decreases monocyte/macrophage infiltration, the expression of the adhesion molecule ICAM-1 and of the proto-oncogene c-*fos* in the kidney and myocardium (Muller *et al.,* 2000). This indicates that NF-$\kappa$B inhibition can provide protection against ANG-II-induced hypertension and related target-organ damage. Furthermore a decreased conversion of NO to peroxynitrite is likely to ensue. The usefulness of this strategy is supported by the fact that (1) the administration of the superoxide-dismutase analog tempol effectively prevents the development of hypertension in spontaneously hypertensive rats (Schnackenberg *et al.,* 1998) and (2) the antioxidant vitamin C improves acethylcholine-mediated forearm vasodilation in essential hypertensive patients (Taddei *et al.,* 1998) and lowers blood pressure in hypertensive patients (Duffy *et al.,* 1999).

Available findings concerning the capacity of the various pharmacological agents to improve NO synthesis are controversial. However, consistent data were obtained as far as $Ca^{2+}$ antagonists are concerned. The vasodilatory response to nitredipine (a typical L-type $Ca^{2+}$-channel blocker dihydropiridine) was first shown to depend, at least in part, upon an intact eNOS activity (Gunther *et al.,* 1992). Further studies have thereafter demonstrated that other dihydropiridine compounds (nifedipine, nimodipine, and nisoldipine) are able to restore NO release from endothelial cells of small resistance arteries (Dhein *et al.,* 1995) and porcine coronary artery (Berkels *et al.,* 1996). Since eNOS is a $Ca^{2+}$-dependent enzyme (see section II.B), the finding of NO release induced by $Ca^{2+}$ antagonists might at first appear to be paradoxical. Furthermore, endothelial cells, although provided with shear stress-activated cation channels, are known to lack L-type $Ca^{2+}$ channels, i.e., the classical target of the dihydropiridine agents (Naruse and Sokabe, 1993). This paradox has been recently clarified by experiments carried out in bovine endothelial cells, which have lead to the conclusion that the dihydropiridine-induced NO release occurs through enhanced $Ca^{2+}$ influx via shear stress-activated cation channels (Salameh *et al.,* 1996). The stimulatory effect of $Ca^{2+}$ antagonists on NO release was subsequently shown to occur also with other dihydropiridines (isradipine, pramidipine, amlodipine, and benidipine) (Akopov, 1996; Mori *et al.,* 1998; Zhang and Hintze, 1998; Kitakaze *et al.,* 1999), as well as with other $Ca^{2+}$ antagonists (verapamil

and diltiazem) (Salameh *et al.,* 1995; Takase *et al.,* 1996; Dhein *et al.,* 1999). It remains, however, unclear whether the well-known antioxidant properties of some of the $Ca^{2+}$ antagonists may be also important for long-term improving "endothelium dysfunction" and NO availability. Two multicenter ongoing trials have been designed to address this issue (Lüscher and Noll, 1996), and their results should be available in the near future.

## V. Concluding Remarks

During the past few years, convincing evidence has been accumulated that the interplay between ET-1 and NO is crucial in the regulation of structure and function of the cardiovascular system not only under physiological conditions, but also and especially under pathological conditions.

A lot of findings support the contention that in hypertension, atherosclerosis, diabetes mellitus, and dyslipidemia, i.e., conditions associated with oxygen free radicals generation, a number of mechanisms are activated, that can lead in the blood vessels to an imbalance between ET-1, which is increased, and NO, which is decreased. The diversity of these mechanisms, as well as their complex interplay with variation of genes involved in the functional regulation of endothelial cells and vascular SMCs, is likely to account for the different individual susceptibility to cardiovascular diseases.

The full elucidation of highly complex molecular mechanisms underlying the regulation of genes involved in ET-1 and NO synthesis is obviously a starting point for planning strategies of prevention and/or correction of the ET- 1/NO imbalance. The possibility of correcting this imbalance pharmacologically is attractive from the therapeutic standpoint, as it might eventually modify our strategies for preventing and treating cardiovascular diseases.

## Acknowledgments

This work was supported by Ministero dell'Università e Ricerca Scientifica Grant COFIN-98 06197882 to GGN. The authors are indebted to Giuseppe Gottardo and Alberta Coi for outstanding secretarial support.

## References

Aji, W., Ravalli, S., Szabolcs, M., Jiang, X. C., Sciacca, R. R., Michler, R. E., and Cannon, P. J. (1997). L-arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* **95,** 430–437.

Akopov, S. E. (1996). Nitric oxide (EDRF) enhances the vasorelaxing effect of dihydropyridine calcium antagonists in isolated human middle cerebral arteries. *Methods Find. Exp. Clin. Pharmacol.* **18,** 101–104.

Aliev, G., Shi, J., Perry, G., Friedland, R. P., and Lamanne, J. C. (2000). Decreased constitutive nitric oxide synthase, but increased inducible nitric oxide synthase and endothelin-1 immunoreactivity in aortic endothelial cells of Donryu rats on cholesterol-enriched diet. *Anat. Rec.* **260,** 16–25.

Alonso, J., Sanchez, D. M., Monton, M., Casado, S., and Lopez-Farre, A. (1997). Endothelial cytosolic proteins bind to the 3′ untranslated region of endothelial nitric oxide synthase mRNA: Regulation by tumor necrosis factor alpha. *Mol. Cell Biol.* **17,** 5719–5726.

Andrew, P. J., and Mayer, B. (1999). Enzymatic function of nitric oxide synthases. *Cardiovasc. Res.* **43,** 521–531.

Balligand, J. L., Ungureanu-Longrois, D., Simmons, W. W., Kobzik, L., Lowenstein, C. J., Lamas, S., Kelly, R. A., Smith, T. W., and Michel, T. (1995). Induction of NO synthase in rat cardiac microvascular endothelial cells by IL-1$\beta$ and INF-gamma. *Am. J. Physiol.* **268,** H1293– H1303.

Banting, J. D., Friberg, P., and Adams, M. A. (1996). Acute hypertension after nitric oxide synthase inhibition is mediated primarily by increased endothelin vasoconstriction. *J. Hypertens.* **14,** 975–981.

Barer, G., Emery, C., Stewart, A., Bee, D., and Howard, P. (1993). Endothelial control of the pulmonary circulation in normal and chronically hypoxic rats. *J. Physiol. (London)* **463,** 1–16.

Barton, M., Haudenschild, C. C., D'Uscio, L. V., Shaw, S., Munter, K., and Lüscher, T. F. (1998a). Endothelin $ET_A$ receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. USA* **95,** 14,367–14,372.

Barton, M., Shaw, S., d'Uscio, L. V., Moreau, P., and Lüscher, T. F. (1998b). Differential modulation of the renal and myocardial endothelin system by angiotensin II *in vivo.* Effects of chronic selective ETA receptor blockade. *J. Cardiovasc. Pharmacol.* **31,** S265–S268.

Battistini, B., Chailler, P., D'Orleans-Juste, P., Brière, N., and Sirois, P. (1993). Growth regulatory properties of endothelins. *Peptides* **14,** 385–399.

Beck, K. F., Mohaupt, M. G., and Sterzel, R. B. (1995). Endothelin-1 inhibits cytokine-stimulated transcription of inducible nitric oxide synthase in glomerular mesangial cells. *Kidney Int.* **48,** 1893–1899.

Beck, K. F., and Sterzel, R. B. (1996). Cloning and sequencing of the proximal promoter of the rat iNOS gene: Activation of NF-$\kappa$B is not sufficient for transcription of the iNOS gene in rat mesangial cells. *FEBS Lett.* **394,** 263–267.

Belloni, A. S., Rossi, G. P., Andreis, P. G., Neri, G., Albertin, G., Pessina, A. C., and Nussdorfer, G. G. (1996). Endothelin adrenocortical secretagogue effect is mediated by the B receptor in rats. *Hypertension* **27,** 1153–1159.

Berkels, R., Bertsch, A., Breitenbach, T., Klaus, W., and Rösen, R. (1996). The calcium-antagonist nifedipine stimulates endothelial NO release in therapeutical concentrations. *Pharmaceut. Pharmacol. Lett.* **6,** 75–78.

Best, P. J., Lerman, L. O., Romero, J. C., Richardson, D., Holmes, D. R. J., and Lerman, A. (1999). Coronary endothelial function is preserved with chronic endothelin receptor antagonism in experimental hypercholesterolemia *in vitro. Arterioscler. Thromb. Vasc. Biol.* **19,** 2769–2775.

Bilsel, A. S., Moini, H., Tetik, E., Aksungar, F., Kaynak, B., and Özer, A. (2000). 17$\beta$-Estradiol modulates endothelin-1 expression and release in human endothelial cells. *Cardiovasc. Res.* **46,** 579–584.

Bishop-Bailey, D. (2000). Peroxisome proliferator-activated receptors in the cardiovascular system. *Br. J. Pharmacol.* **129,** 823–834.

Bloch, K. D. (1999). Regulation of endothelial NO synthase mRNA stability: RNA-binding proteins crowd on the 3′-untranslated region. *Circ. Res.* **85,** 653–655.

Bode-Boger, S. M., Boger, R. H., Alfke, H., Heinzel, D., Tsikas, D., Creutzig, A., Alexander, K., and Frölich, J. C. (1996a). L-Arginine induces nitric oxide-dependent vasodilation in patients with critical limb ischemia. A randomized, controlled study. *Circulation* **93,** 85–90.

Bode-Boger, S. M., Boger, R. H., Creutzig, A., Tsikas, D., Gutzki, F. M., Alexander, K., and Frölich, J. C. (1994). L-Arginine infusion decreases peripheral arterial resistance and inhibits platelet aggregation in healthy subjects. *Clin. Sci.* **87,** 303–310.

Bode-Boger, S. M., Boger, R. H., Kienke, S., Junker, W., and Frölich, J. C. (1996b). Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochem. Biophys. Res. Commun.* **219,** 598–603.

Boger, R. H., Bode-Boger, S. M., Thiele, W., Creutzig, A., Alexander, K., and Frölich, J. C. (1998). Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J. Am. Coll. Cardiol.* **32,** 1336–1344.

Boulanger, C., and Lüscher, T. F. (1990). Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.* **85,** 587–590.

Brandes, R. P., Schmitz-Winnenthal, F. H., Félétou, M., Gödecke, A., Huang, P. L., Vanhoutte, P. M., Fleming, I., and Busse, R. (2000). An endothelium-derived hyperpolarizing factor distinct from NO and prostacyclin is a major endothelium-dependent vasodilator in resistance vessels of wild-type and endothelial NO synthase knockout mice. *Proc. Natl. Acad. Sci. USA* **97,** 9747–9752.

Bredt, D. S., and Snyder, S. H. (1994). Nitric oxide: A physiologic messenger molecule. *Annu. Rev. Biochem.* **63,** 175–195.

Browatzki, M., Schmidt, J., Kubler, W., and Kranzhofer, R. (2000). Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-$\kappa$B in human vascular smooth muscle cells. *Basic Res. Cardiol.* **95,** 98–105.

Buchan, K. W., and Hassal, D. G. (2000). PPAR agonists as direct modulators of the vessel wall in cardiovascular disease. *Med. Res. Rev.* **20,** 350–366.

Burnett, J. C. J. (1997). Coronary endothelial dysfunction in the hypertensive patient: From myocardial ischemia to heart failure. *J. Hum. Hypertens.* **11,** 45–49.

Burnstock, G. (1999). Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J. Anat.* **194,** 335–342.

Buttery, L. D., Springall, D. R., Chester, A. H., Evans, T. J., Standfield, E. N., Parums, D. V., Yacoub, M. H., and Polak, J. M. (1996). Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab. Invest.* **75,** 77–85.

Campbell, A. I., Kuliszewski, M. A., and Stewart, D. J. (1999). Cell-based gene transfer to the pulmonary vasculature: Endothelial nitric oxide synthase overexpression inhibits monocrotaline-induced pulmonary hypertension. *Am. J. Respir. Cell Mol. Biol.* **21,** 567–575.

Cardillo, C., Kilcoyne, C. M., Cannon, R. O., and Panza, J. A. (2000). Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels *in vivo. Hypertension* **3,** 1237–1241.

Chen, Y. F., and Oparil, S. (2000). Endothelin and pulmonary hypertension. *J. Cardiovasc. Pharmacol.* **35,** 49–54.

Cheng, T. H., Shih, N. L., Chen, S. Y., Wang, D. L., and Chen, J. J. (1999). Reactive oxygen species modulate endothelin-1-induced *c-fos* gene expression in cardiomyocytes. *Cardiovasc. Res.* **41,** 654–662.

Cooke, J. P. (1998). Is atherosclerosis an arginine defiency disease? *J. Investig. Med.* **56,** 377–380.

Cooke, J. P., and Tsao, P. S. (1997). Arginine: A new therapy for atherosclerosis? *Circulation* **95,** 311–312.

Cosentino, F., and Lüscher, T. F. (1999). Tetrahydrobiopterin and endothelial nitric oxide synthase activity. *Cardiovasc. Res.* **43,** 274–278.

Dhein, S., Salameh, A., Berkels, R., and Klaus, W. (1999). Dual mode of action of dihydropyridine calcium antagonists: A role for nitric oxide. *Drugs* **58,** 397–404.

Dhein, S., Zhao, Y., Simsek, S., Salameh, A., and Klaus, W. (1995). Actions of 1,4-dihydropyridines in isolated mesenteric vascular beds. *J. Cardiovasc. Pharmacol.* **26,** 784–791.

Delerive, P., Martin-Nizard, F., Chinetti, G., Trottein, F., Fruchart, J. C., Najib, J., Duriez, P., and Staels, B. (1999). Peroxisome proliferator-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. *Circ. Res.* **85,** 394–402.

Doshi, S. N., Goodfellow, J., Lewis, M. J., and McDowell, I. F. (1999). Homocysteine and endothelial function. *Cardiovasc. Res.* **42,** 578–582.

Drexler, H., and Hornig, B. (1999). Endothelial dysfunction in human disease. *J. Mol. Cell Cardiol.* **31,** 51–60.

Drexler, H., and Zeiher, A. M. (1991). Endothelial function in human coronary arteries *in vivo*. Focus on hypercholesterolemia. *Hypertension* **18,** II90–II99.

Drummond, G. R., Cai, H., Davis, M. E., Ramasamy, S., and Harrison, D. G. (2000). Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ. Res.* **86,** 347–354.

Duerrschmidt, N., Wippich, N., Goettsch, W., Broemme, H. J., and Morawietz, H. (2000). Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. *Biochem. Biophys. Res. Commun.* **269,** 713–717.

Duffy, S. J., Gokce, N., Holbrook, M., Huang, A., Frei, B., Keaney, J. F. J., and Vita, J. A. (1999). Treatment of hypertension with ascorbic acid. *Lancet* **354,** 2048–2049.

Dupuis, J., Cernacek, P., Tardif, J. C., Stewart, D. J., Gosselin, G., Dyrda, I., Bonan, R., and Crepeau, J. (1998). Reduced pulmonary clearance of endothelin-1 in pulmonary hypertension. *Am. Heart J.* **135,** 614–620.

Eikelboom, J. W., Lonn, E., Genest, J. J., Hankey, G., and Yusuf, S. (1999). Homocyst(e)ine and cardiovascular disease: A critical review of the epidemiologic evidence. *Ann. Intern. Med.* **131,** 363–375.

Emori, T., Hirata, Y., Imai, T., Ohta, K., Kanno, K., Eguchi, S., and Marumo, F. (1992). Cellular mechanism of thrombin on endothelin-1 biosynthesis and release in bovine endothelial cell. *Biochem. Soc. Trans.* **44,** 2409–2411.

Emori, T., Hirata, Y., Ohta, K., Kanno, K., Eguchi, S., Imai, T., Shichiri, M., and Marumo, F. (1991). Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension* **18,** 165–170.

Fadel, B. M., Boutet, S. C., and Quertermous, T. (2000). Endothelial cell-specific regulation of the murine endothelin-1 gene. *J. Cardiovasc. Pharmacol.* **35,** 7–11.

Fei, J., Viedt, C., Soto, U., Elsing, C., Jahn, L., and Kreuzer, J. (2000). Endothelin-1 and smooth muscle cells: Induction of jun amino-terminal kinase through an oxygen radical-sensitive mechanism. *Arterioscler. Thromb. Vasc. Biol.* **20,** 1244–1249.

Fernandez-Patron, C., Radomski, M. W., and Davidge, S. T. (1999). Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ. Res.* **85,** 906–911.

Fleming, I., and Busse, R. (1999). NO: The primary EDRF. *J. Mol. Cell Cardiol.* **31,** 5–14.

Förstermann, U., Boissel, J. P., and Kleinert, H. (1998). Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J.* **12,** 773–790.

Gallois, C., Habib, A., Tao, J., Moulin, S., Maclouf, J., Mallat, A., and Lotersztajn, S. (1998). Role of NF-kB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells. Involvement of cyclooxygenase-2. *J. Biol. Chem.* **273,** 23183–23190.

Gan, L. M., Selin-Sjogren, L., Doroudi, R., and Jern, S. (2000). Temporal regulation of endothelial ET-1 and eNOS expression in intact human conduit vessels exposed to different intraluminal pressure levels at physiological shear stress. *Cardiovasc. Res.* **48,** 168–177.

Gellai, M., Fletcher, T., Pullen, M., and Nambi, P. (1996). Evidence for the existence of endothelin-B receptor subtypes and their physiological roles in the rat. *Am. J. Physiol.* **271,** R254–R261.

Giaid, A., Michel, R. P., Stewart, D. J., Sheppard, M., Corrin, B., and Hamid, Q. (1993). Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. *Lancet* **341,** 1550–1554.

Giaid, A., Saleh, D., Yanagisawa, M., and Forbes, R. D. (1995). Endothelin-1 immunoreactivity and mRNA in the transplanted human heart. *Transplantation* **59,** 1308–1313.

Goldie, R. G. (1999). Endothelins in health and disease: An overview. *Clin. Exp. Pharmacol. Physiol.* **26,** 145–148.

Goligorsky, M. S., Budzikowski, A. S., Tsukahara, H., and Noiri, E. (1999). Co-operation between endothelin and nitric oxide in promoting endothelial cell migration and angiogenesis. *Clin. Exp. Pharmacol. Physiol.* **26,** 269–271.

Gray, G. A., and Webb, D. J. (1996). The endothelin system and its potential as a therapeutic target in cardiovascular disease. *Pharmacol. Ther.* **72,** 109–148.

Gunther, J., Dhein, S., Rosen, R., Klaus, W., and Fricke, U. (1992). Nitric oxide (EDRF) enhances the vasorelaxing effect of nitrendipine in various isolated arteries. *Basic Res. Cardiol.* **87,** 452–460.

Hampl, V., and Herget, J. (2000). Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol. Rev.* **80,** 1337–1372.

Hasdai, D., Holmes, D. R. J., Garratt, K. N., Edwards, W. D., and Lerman, A. (1997). Mechanical pressure and stretch release endothelin-1 from human atherosclerotic coronary arteries *in vivo*. *Circulation* **95,** 357–362.

Hinson, J. P., Kapas, S., and Smith, D. M. (2000). Adrenomedullin, a multifunctional regulatory peptide. *Endocr. Rev.* **21,** 138–167.

Hinson, J. P., Cameron, L. A., and Kapas, S. (1996). Regulation of adrenal vasculature tone: Role of endothelin-1 and nitric oxide. *Endocr. Res.* **22,** 875–879.

Hinson, J. P., Vinson, G. P., Kapas, S., and Teja, R. (1991). The relationship between adrenal vascular events and steroid secretion: The role of mastcells and endothelin. *J. Steroid Biochem. Mol. Biol.* **40,** 381–389.

Hirahashi, J., Nakaki, T., Hishikawa, K., Marumo, T., Yasumori, T., Hayashi, M., Suzuki, H., and Saruta, T. (1996). Endothelin-1 inhibits induction of nitric oxide synthase and GTP cyclohydrolase-I in rat mesangial cells. *Pharmacology* **53,** 241–249.

Hirata, Y., Hayakawa, H., Suzuki, E., Kimura, K., Kikuchi, K., Nagano, T., Hirobe, M., and Omata, M. (1995). Direct measurements of endothelium-derived nitric oxide release by stimulation of endothelin receptors in rat kidney and its alteration in salt-induced hypertension. *Circulation* **91,** 1229–1235.

Hocher, B., Thöne-Reineke, C., Bauer C., Raschack, M., and Neumayer, H. H. (1997). The paracrine endothelin system: Pathophysiology and implications in clinical medicine. *Eur. J. Clin. Chem. Clin. Biochem.* **35,** 175–189.

Hughes, A. K., Stricklett, P. K., Padilla, E., and Kohan, D. E. (1996). Effect of reactive oxygen species on endothelin-1 production by human mesangial cells. *Kidney Int.* **49,** 181–189.

Ignarro, L. J. (1999). Nitric oxide: A unique endogenous signaling molecule in vascular biology. *Biosci. Rep.* **19,** 51–71.

Ikeda, U., Shimpo, M., Murakami, Y., and Shimada, K. (2000). Peroxisome proliferator-activated receptor-gamma ligands inhibit nitric oxide synthesis in vascular smooth muscle cells. *Hypertension* **35,** 1232–1236.

Ikeda, U., Yamamoto, K., Maeda, Y., Shimpo, M., Kanbe, T., and Shimada, K. (1997). Endothelin-1 inhibits nitric oxide synthesis in vascular smooth muscle cells. *Hypertension* **29,** 65–69.

Imai, T., Hirata, Y., Emori, T., Yanagisawa, M., Masaki, T., and Marumo, F. (1992). Induction of endothelin-1 gene by angiotension and vasopression in endothelial cells. *Hypertension* **19,** 753–757.

Inui, D., Yoshizumi, M., Okishima N., Houchi, H., Tsuchiya, K., Kido, H., and Tamaki, T. (1999). Mechanism of endothelin-1(1–31)-induced calcium signaling in human coronary artery smooth muscle cells. *Am. J. Physiol.* **276,** E1067–E1072.

Ishigami, M., Swertjeger, D. K., Hui, M. S., Grenholm, N. A., and Hui, D. Y. (2000). Apolipoprotein E inhibition of vascular smooth muscle cell proliferation, but not the inhibition of migration, is mediated through the activation of inducible nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* **20,** 1020–1026.

Ishizuka, T., Takamizawa-Matsumoto, M., Suzuki, K., and Kurita, A. (1999). Endothelin-1 enhances vascular cell adhesion molecule-1 expression in tumor necrosis factor alpha-stimulated vascular endothelial cells. *Eur. J. Pharmacol.* **369,** 237–245.

Iwashina, M., Hirata, Y., Imai, T., Sato, K., and Marumo, F. (1996). Molecular cloning of endothelial, inducible nitric oxide synthase gene from rat aortic endothelial cell. *Eur. J. Biochem.* **237,** 668–673.

Jeremy, J. Y., Rowe, D., Emsley, A. M., and Newby, A. C. (1999). Nitric oxide and the proliferation of vascular smooth muscle cells. *Cardiovasc. Res.* **43,** 580–594.

Jin, D., Takai, S., Shiota, N., and Miyazaki, M. (1998). Roles of vascular angiotensin converting enzyme and chymase in two-kidney, one clip hypertensive hamsters. *J. Hypertens.* **16,** 657–664.

Joly, G. A., Schini, V. B., and Vanhoutte, P. M. (1992). Balloon injury and interleukin-1 beta induce nitric oxide synthase activity in rat carotid arteries. *Circ. Res.* **71,** 331–338.

Kaburagi, S., Hasegawa, K., Morimoto, T., Araki, M., Sawamura, T., Masaki, T., and Sasayama, S. (1999). The role of endothelin-converting enzyme-1 in the development of $\alpha$1-adrenergic-stimulated hypertrophy in cultured neonatal rat cardiac myocytes. *Circulation* **99,** 292–298.

Kanno, K., Hirata, Y., Imai, T., Iwashina, M., and Marumo, F. (1994). Regulation of inducible nitric oxide synthase gene by interleukin-1$\beta$ in rat vascular endothelial cells. *Am. J. Physiol.* **267,** H2318–H2324.

Kennedy, R. L., Haynes, W. G., and Webb, D. J. (1993). Endothelins as regulators of growth and function in endocrine tissues. *Clin. Endocrinol.* **39,** 259–265.

Kiowski, W., Sutsch, G., Hunziker, P., Muller, P., Kim, J., Oechslin, E., Schmidt, R., Jones, R., and Bertel, O. (1995). Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet* **346,** 732–736.

Kirchengast, M., and Münter, K. (1999). Endothelin-1 and endothelin receptor antagonists in cardiovascular remodeling. *Proc. Soc. Exp. Biol. Med.* **221,** 312–325.

Kishi, F., Minami, K., Okishima, N., Murakami, M., Mori, S., Yano, M., Niwa, Y., Nakaya, Y., and Kido, H. (1998). Novel 31-amino acid lenght endothelins cause constriction of vascular smooth muscle. *Biochem. Biophys. Res. Commun.* **248,** 387–390.

Kitakaze, M., Node, K., Minamino, T., Asanuma, H., Kuzuya, T., and Hori, M. (1999). A $Ca^{2+}$ channel blocker, benidipine, increases coronary blood flow and attenuates the severity of myocardial ischemia via NO-dependent mechanisms in dogs. *J. Am. Coll. Cardiol.* **33,** 242–249.

Kone, B. C. (2000). Protein–protein interactions controlling nitric oxide synthases. *Acta Physiol. Scand.* **168,** 27–31.

Krum, H., Viskoper, R. J., Lacourcière, Y., Budde, M., and Charlon, V. (1998). The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. *N. Engl. J. Med.* **338,** 784–790.

Lerman, A., Edwards, B. S., Hallett, J. W., Heublein, D. M., Sandberg, S. M., and Burnett, J. C. J. (1991). Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N. Engl. J. Med.* **325,** 997–1001.

Lerman, A., Holmes, D. R., Jr., Bell, M. R., Garratt, K. N., Nishimura, R. A., and Burnett, J. C., Jr. (1995). Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation* **92,** 2426–2431.

Li, H., and Föstermann, U. (2000). Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* **190,** 244–254.

Linder, L., Kiowski, W., Buhler, F. R., and Lüscher, T. F. (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation *in vivo.* Blunted response in essential hypertension. *Circulation* **81,** 1762–1767.

Lopez-Ongil, S., Hernandez-Perera, O., Navarro-Antolin, J., Perez, D. L., Rodriguez-Puyol, M., Lamas, S., and Rodriguez-Puyol, D. (1998). Role of reactive oxygen species in the signalling cascade of cyclosporine A-mediated up-regulation of eNOS in vascular endothelial cells. *Br. J. Pharmacol.* **124,** 447–454.

Lopez-Ongil, S., Senchak, V., Saura, M., Zaragoza, C., Ames, M., Ballermann, B., Rodriguez-Puyol, M., Rodriguez-Puyol, D., and Lowenstein, C. J. (2000). Superoxide regulation of endothelin-converting enzyme. *J. Biol. Chem.* **275,** 26,423–26,435.

Lüscher, T. F., and Barton, M. (1997). Biology of the endothelium. *Clin. Cardiol.* **20,** II–10. [Abstract]

Lüscher, T. F., and Noll, G. (1996). Endothelial function as an end-point in interventional trials: Concepts, methods and current data. *J. Hypertens.* **14,** S111–S119.

MacAllister, R. J., Rambausek, M. H., Vallance, P., Williams, D., Hoffmann, K. H., and Ritz, E. (1996). Concentration of dimethyl-L-arginine in the plasma of patients with end-stage renal failure. *Nephrol. Dial. Transplant.* **11,** 2449–2452.

Marletta, M. A., Hurshman, A. R., and Rusche, K. M. (1998). Catalysis by nitric oxide synthase. *Curr. Opin. Chem. Biol.* **2,** 656–663.

Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. J., Hall, A. V., Shi, X. M., Tsui, L. C., and Schappert, K. T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem.* **268,** 17478–17488.

Matsuoka, H., Itoh, S., Kimoto, M., Kohno, K., Tamai, O., Wada, Y., Yasukawa, H. X. I. G., Okuda, S., and Imaizumi, T. (1997). Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* **29,** 242–247.

Mazzocchi, G., Malendowicz, L. K., Musajo, F., Gottardo, G., Markowska, A., and Nussdorfer, G. G. (1998). Role of endothelins in regulation of vascular tone in the *in situ* perfused rat adrenals. *Am. J. Physiol.* **274,** E1–E5.

Mazzocchi, G., Rossi G. P., Malendowicz, L. K., Champion, H. C., and Nussdorfer, G. G. (2001). Endothelin-1[1–31], acting as an ETA-receptor selective agonist, stimulates proliferation of cultured rat zona glomerulosa cells. *FEBS Lett.* **487,** 194–198.

Mazzocchi, G., Rossi, G. P., Rebuffat, P., Malendowicz, L. K., Markowska, A., and Nussdorfer, G. G. (1997). Endothelin-1 stimulates deoxyribonucleic acid synthesis and cell proliferation in rat adrenal zona glomerulosa, acting through an endothelin A receptor coupled with protein kinase C- and tyrosine kinase-dependent signaling pathways. *Endocrinology* **138,** 2333–2338.

McIntyre, M., Bohr, D. F., and Dominiczak, A. F. (1999). Endothelial function in hypertension: The role of superoxide anion. *Hypertension* **34,** 539–545.

McIntyre, M., MacFadyen, R. J., Meredith, P. A., Brouard, R., and Reid, J. L. (1996). Dose-ranging study of the angiotensin II receptor antagonist irbesartan (SR 47436/BMS-186295) on blood pressure and neurohormonal effects in salt-depleted men. *J. Cardiovasc. Pharmacol.* **28,** 101–106.

Mehta, S., Stewart, D. J., Langleben, D., and Levy, R. D. (1995). Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. *Circulation* **92,** 1539–1545.

Minchenko, A., and Caro, J. (2000). Regulation of endothelin-1 gene expression in human microvascular endothelial cells by hypoxia and cobalt: Role of hypoxia responsive element. *Mol. Cell. Biochem.* **208,** 53–62.

Ming, Z., Parent, R., Thorin, E., and Lavallee, M. (1998). Endothelin-dependent tone limits acetylcholine-induced dilation of resistance coronary vessels after blockade of NO formation in conscious dogs. *Hypertension* **32,** 844–848.

Miyauchi, T., and Masaki, T. (1999). Pathophysiology of endothelin in the cardiovascular system. *Annu. Rev. Physiol.* **61,** 391–415.

Mombouli, J. V., and Vanhoutte, P. M. (1999). Endothelial dysfunction: From physiology to therapy. *J. Mol. Cell Cardiol.* **31,** 61–74.

Morawietz, H., Talanow, R., Szibor, M., Rueckschloss, U., Schubert, A., Bartling, B., Darmer, D., and Holtz, J. (2000). Regulation of the endothelin system by shear stress in human endothelial cells. *J. Physiol. (London)* **525,** 761–770.

Moreau, P., Takase, H., Kung, C. F., van Rooijen, M. M., Schaffner, T., and Lüscher, T. F. (1995). Structure and function of the rat basilar artery during chronic nitric oxide synthase inhibition. *Stroke* **26,** 1922–1928.

Mori, T., Takeuchi, T., Ohura, M., Miyakoda, G., Fujiki, H., Orito, K., Yoshida, K., Hirano, T., Yamamura, Y., Sumida, T., Nakaya, Y., Satake, H., and Hata, F. (1998). Pranidipine, a new 1, 4-dihydropyridine calcium channel blocker, enhances cyclic GMP-independent nitric oxide-induced relaxation of the rat aorta. *Mol. Cell. Biochem.* **178,** 335–343.

Morris, S. M., and Billiar, T. R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.* **266,** E829–E839.

Muller, D. N., Dechend, R., Mervaala, E. M., Park, J. K., Schmidt, F., Fiebeler, A., Theuer, J., Breu, V.,

Ganten, D., Haller, H., and Luft, F. C. (2000). NF-$\kappa$B inhibition ameliorates angiotensin II-induced inflammatory damage in rats. *Hypertension* **35,** 193–201.

Nakano, A., Kishi, F., Minami, K., Wakabayashi, H., Nakaya, Y., and Kido, H. (1997). Selective conversion of big endothelins to tracheal smooth muscle-constricting 31-amino acid-length endothelins by chymase from human mastcells. *J. Immunol.* **159,** 1987–1992.

Nakayama, M., Yasue, H., Yoshimura, M., Shimasaki, Y., Kugiyama, K., Ogawa, H., Motoyama, T., Saito, Y., Ogawa, Y., Miyamoto, Y., and Nakao, K. (1999). $T^{-786} \rightarrow C$ mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* **99,** 2864–2870.

Namiki, A., Hirata, Y., Ishikawa, M., Moroi, M., Aikawa, J., and Machii, K. (1992). Endothelin-1- and endothelin-3-induced vasorelaxation via common generation of endothelium-derived nitric oxide. *Life Sci.* **50,** 677–682.

Naruse, K., and Sokabe, M. (1993). Involvement of stretch-activated ion channels in $Ca^{2+}$ mobilization to mechanical stretch in endothelial cells. *Am J. Physiol.* **264,** C1037–C1044.

Navarro-Antolin, J., Hernandez-Perera, O., Lopez-Ongil, S., Rodriguez-Puyol, M., Rodriguez-Puyol, D., and Lamas, S. (1998). CsA and FK506 up-regulate eNOS expression: Role of reactive oxygen species and AP-1. *Kidney Int.* **68,** S20–S24.

Nussdorfer, G. G., Rossi, G. P., and Belloni, A. S. (1997). The role of endothelins in the paracrine control of the secretion and growth of the adrenal cortex. *Int. Rev. Cytol.* **171,** 267–308.

Nussdorfer, G. G., Rossi, G. P., Malendowicz, L. K., and Mazzocchi, G. (1999). Autocrine-paracrine endothelin system in the physiology and pathology of steroid-secreting tissues. *Pharmacol. Rev.* **51,** 403–438.

Oemar, B. S., Tschudi, M. R., Godoy, N., Brovkovich, V., Malinski, T., and Lüscher, T. F. (1998). Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* **97,** 2494–2498.

Ohta, K., Hirata, Y., Imai, T., Kanno, K., Emori, T., Shichiri, M., and Marumo, F. (1990). Cytokine-induced release of endothelin-1 from porcine renal epithelial cell line. *Biochem. Biophys. Res. Commun.* **169,** 578–584.

Owada, A., Tomita, K., Terada, Y., Sakamoto, H., Nonoguchi, H., and Marumo, F. (1994). Endothelin (ET)-3 stimulates cyclic guanosine 3′,5′-monophosphate production via $ET_B$ receptors by producing nitric oxide in isolated rat glomerulus, and in cultured rat mesangial cells. *J. Clin. Invest.* **93,** 556–563.

Pacheco, M. E., Marin, J., Manso, A. M., Rodriguez-Martinez, M. A., Briones, A., Salaices, M., and Redondo, J. (2000). Nitric oxide synthase induction by ouabain in vascular smooth muscle cells from normotensive and hypertensive rats. *J. Hypertens.* **18,** 877–884.

Panza, J. A., Quyyumi, A. A., Brush, J. E. J., and Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N. Engl. J. Med.* **323,** 22–27.

Papapetropoulos, A., Rudic, R. D., and Sessa, W. C. (1999). Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovasc. Res.* **43,** 509–520.

Patel, R. P., Levonen, A. L., Crawford, J. H., and Darley-Usmar, V. M. (2000). Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc. Res.* **47,** 465–474.

Peng, H. B., Libby, P., and Liao, J. K. (1995). Induction and stabilization of I-$\kappa$B $\alpha$ by nitric oxide mediates inhibition of NF-$\kappa$B. *J. Biol. Chem.* **270,** 14214–14219.

Quehenberger, P., Bierhaus, A., Fashing, P., Muellner, C., Klevesath, M., Hong, M., Stier, G., Sattler, M., Schleicher, E., Speiser, W., and Nawroth, P. P. (2000). Endothelin-1 transcription is controlled by nuclear factor-$\kappa$B in AGE-stimulated cultured endothelial cells. *Diabetes* **49,** 1561–1570.

Quyyumi, A. A., Mulcahy, D., Andrews, N. P., Husain, S., Panza, J. A., and Cannon, R. O. (1997). Coronary vascular nitric oxide activity in hypertension and hypercholesterolemia. Comparison of acetylcholine and substance P. *Circulation* **95,** 104–110.

Rizvi, M. A., and Myers, P. R. (1997). Nitric oxide modulates basal and endothelin-induced coronary artery vascular smooth muscle cell proliferation and collagen levels. *J. Mol. Cell Cardiol.* **29,** 1779–1789.

Robert-Thomson, S. J. (2000). Peroxisome proliferator-activated receptors in tumorigenesis: Target of tumor promotion and treatment. *Immunol. Cell. Biol.* **78,** 436–441.

Robinson, L. J., Weremowicz, S., Morton, C. C., and Michel, T. (1994). Isolation and chromosomal localization of the human endothelial nitric oxide synthase (NOS III) gene. *Genomics* **19,** 350–357.

Rocha, R., Stier, C. T., Kifor, I., Ochoa-Maye, M. R., Rennke, H. G., Williams, G. H., and Adler, G. K. (2000). Aldosterone: A mediator of myocardial necrosis and renal arteriopathy. *Endocrinology* **141,** 3871–3878.

Rossi, G. P., Albertin, G., Bova, S., Belloni, A. S., Fallo, F., Pagotto, U., Trevisi, L., Palù, G., Pessina, A. C., and Nussdorfer, G. G. (1997a). Autocrine-paracrine role of endothelin-1 in the regulation of aldosterone synthase expression and intracellular $Ca^{2+}$ in human adrenocortical carcinoma. *Endocrinology* **138,** 4421–4426.

Rossi, G. P., Albertin, G., Neri, G., Andreis, P. G., Hofmann, S., Pessina, A. C., and Nussdorfer, G. G. (1997b). Endothelin-1 stimulates steroid secretion of human adrenocortical cells *ex vivo* via both $ET_A$ and $ET_B$ receptor subtypes. *J. Clin. Endocrinol. Metab.* **82,** 3445–3449.

Rossi, G. P., Belloni, A. S., Nussdorfer, G. G., and Pessina, A. C. (2000a). Endothelin-1 and the adrenal gland. *J. Cardiovasc. Pharmacol.* **35,** 18–20.

Rossi, G. P., Colonna, S., Pavan, E., Albertin, G., Della Rocca, F., Gerosa, G., Casarotto, D., Sartore, S., Pauletto, P., and Pessina, A. C. (1999). Endothelin-1 and its mRNA in the wall layers of human arteries *ex vivo. Circulation* **99,** 1147–1155.

Rossi, G. P., Seccia, T. M., Albertin, G., and Pessina, A. C. (2000b). Measurement of endothelin: Clinical and research use. *Ann. Clin. Biochem.* **37,** 608–626.

Rubanyi, G. M., and Botelho, L. H. (1991). Endothelins. *FASEB J.* **5,** 2713–2720.

Rubanyi, G. M., and Polokoff, M. A. (1994). Endothelins: Molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.* **46,** 325–415.

Rubino, A., Loesch, A., and Burnstock, G. (1999). Nitric oxide and endothelin-1 in coronary and pulmonary circulation. *Int. Rev. Cytol.* **189,** 59–93.

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., and Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J. Clin. Invest.* **101,** 731–736.

Russell, F. D., and Davenport, A. P. (1999). Secretory pathways in endothelin synthesis. *Br. J. Pharmacol.* **126,** 391–398.

Russell, F. D., and Molenaar, P. (2000). The human heart endothelin system: ET-1 synthesis, storage, release and effect. *Trends Pharmacol. Sci.* **21,** 353–359.

Saijonmaa, O., Nyman, T., Hohenthal, U., and Fyhrquist, F. (1991). Endothelin-1 is expressed and released by a human endothelial hybrid cell line (EA.hy-926). *Biochem. Biophys. Res. Commun.* **181,** 529–536.

Salameh, A., Eisert, A., Schomecker, G., Dhein, S., and Klaus, W. (1995). Effects of nitrendipine, dotarizine and diltiazem on intracellular calcium in cultured bovine aortic endothelial cells. *Pharmacol. Res.* **31,** 227. [Abstract]

Salameh, A., Schomecker, G., Breitkopf, K., Dhein, S., and Klaus, W. (1996). The effect of the calcium-antagonist nitrendipine on intracellular calcium concentration in endothelial cells. *Br. J. Pharmacol.* **118,** 1899–1904.

Salomone, O. A., Elliott, P. M., Calvino, R., Holt, D., and Kaski, J. C. (1996). Plasma immunoreactive endothelin concentration correlates with severity of coronary artery disease in patients with stable angina pectoris and normal ventricular function. *J. Am. Coll. Cardiol.* **28,** 14–19.

Samson, W. K. (1999). Adrenomedullin and the control of fluid and electrolyte homeostasis. *Annu. Rev. Physiol.* **61,** 363–390.

Sanker, S., Chandrasekharan, U. M., Wilk, D., Glynias, M. J., Karnik, S. S., and Husain, A. (1997). Distinct multisite synergistic interactions determine substrate specificities of human chymase and rat chymase-1 for angiotensin II formation and degradation. *J. Biol. Chem.* **272,** 2963–2968.

Sato, J., Nair, K., Hiddinga, J., Eberhardt, N. L., Fitzpatrick, L. A., Katusic, Z. S., and O'Brien, T. (2000).

eNOS gene transfer to vascular smooth muscle cells inhibits cell proliferation via upregulation of p27 and p21 and not apoptosis. *Cardiovasc. Res.* **47,** 697–706.

Schnackenberg, C. G. Welch, W. J., and Wilcox, C. S. (1998). Normalization of blood and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: Role of nitric oxide. *Hypertension* **32,** 59–64.

Setoguchi, S., Mohri, M., Hirooka, Y., and Ichiki, T. (1999). Intracoronary tetrahydrobiopterin improves endothelial dysfunction in patients with coronary risk factors. *Circulation* **100,** 7. [Abstract]

Shesely, E. G., Maeda, N., Kim, H. S., Desai, K. M., Krege, J. H., Laubach, V. E., Sherman, P. A., Sessa, W. C., and Smithies, O. (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **93,** 13,176–13,181.

Shichiri, M., Yokokura, M., Marumo, F., and Hirata, Y. (2000). Endothelin-1 inhibits apoptosis of vascular smooth muscle cells induced by nitric oxide and serum deprivation via MAP kinase pathway. *Arterioscler. Thromb. Vasc. Biol.* **20,** 989–997.

Sutsch, G., and Kiowski, W. (2000). Endothelin and endothelin receptor antagonism in heart failure. *J. Cardiovasc. Pharmacol.* **35,** S69–S73.

Taddei, S., Mattei, P., Virdis, A., Sudano, I., Ghiadoni, L., and Salvetti, A. (1993a). Forearm vasodilation in response to acetylcholine is increased by potassium in essential hypertensive patients. *J. Hypertens.* **11,** S144–S145.

Taddei, S., and Salvetti, A. (1997). Endothelial dysfunction in hypertension. *Adv. Exp. Med. Biol.* **432,** 235–246.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., and Salvetti, A. (1998). Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* **97,** 2222–2229.

Taddei, S., Virdis, A., Ghiadoni, L., Sudano, I., Notari, M., and Salvetti, A. (1999). Vasoconstriction to endogenous endothelin-1 is increased in the peripheral circulation of patients with essential hypertension. *Circulation* **100,** 1680–1683.

Taddei, S., Virdis, A., Mattei, P., and Salvetti, A. (1993b). Vasodilation to acetylcholine in primary and secondary forms of human hypertension. *Hypertension* **21,** 929–933.

Takada, K., Matsumura, Y., Dohmen, S., Mitsutomi, N., Takaoka, M., and Morimoto, S. (1996). Endothelin-1 secretion from cultured vascular endothelial cells of DOCA-salt hypertensive rats. *Life Sci.* **59,** PL111–PL116.

Takase, H., Moreau, P., Kung, C. F., Nava, E., and Lüscher, T. F. (1996). Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency. Effect of verapamil and trandolapril. *Hypertension* **27,** 25–31.

Teichert, A. M., Karantzoulis-Fegaras, F., Wang, Y., Mawji, I. A., Bei, X., Gnanapandithen, K., and Marsden, P. A. (1998). Characterization of the murine endothelial nitric oxide synthase promoter. *Biochim. Biophys. Acta* **1443,** 352–357.

Tharaux, P. L., Chatziantoniou, C., Casellas, D., Fouassier, L., Ardaillou, R., and Dussaule, J. C. (1999). Vascular endothelin-1 gene expression and synthesis and effect on renal type I collagen synthesis and nephroangiosclerosis during nitric oxide synthase inhibition in rats. *Circulation* **99,** 2185–2191.

Tomasian, D., Keaney, J. F., Jr., and Vita, J. A. (2000). Antioxidants and the bioactivity of endothelium-derived nitric oxide. *Cardiovasc. Res.* **47,** 426–435.

Tsukahara, H., Ende, H., Magazine, H. I., Bahou, W. F., and Goligorsky, M. S. (1994). Molecular and functional characterization of the non-isopeptide-selective $ET_B$ receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *J. Biol. Chem.* **269,** 21,778–21,785.

Urata, H., Kinoshita, A., Misono, K. S., Bumpus, F. M., and Husain, A. (1990). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J. Biol. Chem.* **265,** 22,348–22,357.

Vallance, P., Leone, A., Calver, A., Collier, J., and Moncada, S. (1992). *J. Cardiovasc. Pharmacol.* **20,** S60–S62.

Vanhoutte, P. M. (1996). Endothelial dysfunction in hypertension. *J. Hypertens.* **14,** S83–S93.

Venema, R. C., Nishida, K., Alexander, R. W., Harrison, D. G., and Murphy, T. J. (1994). Organization of the bovine gene encoding the endothelial nitric oxide synthase. *Biochim. Biophys. Acta* **1218,** 413–420.

Verhaar, M. C., Strachan, F. E., Newby, D. E., Cruden, N. L., Koomans, H. A., Rabelink, T. J., and Webb, D. J. (1998). Endothelin-A receptor antagonist-mediated vasodilation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* **97,** 752–756.

Wagner, O. F., Christ, G., Wojta, J., Vierhapper, H., Parzer, S., Nowotny, P. J., Schneider, B., Waldhausl, W., and Binder, B. R. (1992). Polar secretion of endothelin-1 by cultured endothelial cells. *J. Biol. Chem.* **267,** 16,066–16,068.

Wang, D. L., Wung, B. S., Peng, Y. C., and Wang, J. J. (1995). Mechanical strain increases endothelin-1 gene expression via protein kinase C pathway in human endothelial cells. *J. Cell Physiol.* **163,** 400–406.

Warner, T. D. (1999). Relationships between the endothelin and nitric oxide pathways. *Clin. Exp. Pharmacol. Physiol.* **26,** 247–252.

Weber, K. T., Armstrong, P. W., Brilla, C. G., Burnett, J. C. J., Cruickshank, J. M., Devereux, R. B., Giles, T. D., Korsgaard, N., Leier, C. V., Mendelsohn, F. A. O., Motz, W. H., Mulvany, M. J., and Strauer, B. E. (1992). Remodeling and reparation of the cardiovascular system. *J. Am. Coll. Cardiol.* **20,** 3–16.

Wever, R. M., Lüscher, T. F., Cosentino, F., and Rabelink, T. J. (1998). Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* **97,** 108–112.

Wilson, S. H., Caplice, N. M., Simari, R. D., Holmes, D. R. J., Carlson, P. J., and Lerman, A. (2000). Activated nuclear factor-$\kappa$B is present in the coronary vasculature in experimental hypercholesterolemia. *Atherosclerosis* **148,** 23–30.

Winkles, J. A., Alberts, G. F., Brogi, E., and Libby, P. (1993). Endothelin-1 and endothelin receptor mRNA expression in normal and atherosclerotic human arteries. *Biochem. Biophys. Res. Commun.* **191,** 1081–1088.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (London)* **332,** 411–415.

Yoshizumi, M., Inui, D., Okishima, N., Houchi, H., Tsuchiya, K., Wakabayashi, H., Kido, H., and Tamaki, T. (1998a). Endothelin-1(1–31), a novel vasoactive peptide, increase $[Ca^{2+}]$i in human coronary artery smooth muscle cells. *Eur. J. Pharmacol.* **348,** 305–309.

Yoshizumi, M., Kim, S., Kagami, S., Hamaguchi, A., Tsuchiya, K., Houchi, H., Iwao, H., Kido, H., and Tamaki, T. (1998b). Effect of endothelin-1(1–31) on extracellular signal-regulated kinases and proliferation of human coronary artery smooth muscle cells. *Br. J. Pharmacol.* **125,** 1019–1027.

Young, M., and Funder, J. W. (2000). Aldosterone and the heart. *Trends Endocrinol. Metab.* **11,** 224–226.

Zamora, M. A., Dempsey, E. C., Walchak, S. J., and Stelzner, T. J. (1993). BQ-123, an ETA receptor antagonist, inhibits endothelin-1-mediated proliferation of human pulmonary artery smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* **9,** 429–433.

Zeiher, A. M., Drexler, H., Saurbier, B., and Just, H. (1993). Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J. Clin. Invest.* **92,** 652–662.

Zeiher, A. M., Drexler, H., Wollschlager, H., and Just, H. (1991). Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. *Circulation* **84,** 1984–1992.

Zhang, X., and Hintze, T. H. (1998). Amlodipine releases nitric oxide from canine coronary microvessels: An unexpected mechanism of action of a calcium channel-blocking agent. *Circulation* **97,** 576–580.

# INDEX

## A



## B

## C

## G



## H



## I



## J



## K



## L

## M



## N



## O

## P



## Q



## R

## S

## T



## U



## V



## W



## X



## Y



## Z