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Kwang W. Jeon

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Paternal Contributions to the Mammalian Zygote: Fertilization after Sperm–Egg Fusion

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Mammalian fertilization has traditionally been regarded as a simple blending of two gametes, during which the haploid genome of the fertilizing spermatozoon constitutes the primary paternal contribution to the resulting embryo. In contrast to this view, new research provides evidence of important cytoplasmic contributions made by the fertilizing spermatozoon to the zygotic makeup, to the organization of preimplantation development, and even reproductive success of new forms of assisted fertilization. The central role of the sperm-contributed centriole in the reconstitution of zygotic centrosome has been established in most mammalian species and is put in contrast with strictly maternal centrosomal inheritance in rodents. The complementary reduction or multiplication of sperm and oocyte organelles during gametogenesis, exemplified by the differences in the biogenesis of centrosome in sperm and oocytes, represents an intriguing mechanism for avoiding their redundancy during early embryogenesis. New studies on perinuclear theca of sperm revealed its importance for both spermatogenesis and fertilization. Remodeling of the sperm chromatin into a male pronucleus is guided by oocyte-produced, reducing peptide glutathione and a number of molecules required for the reconstitution of the functional nuclear envelope and nuclear skeleton. Although some of the sperm structures are transformed into zygotic components, the elimination of others is vital to early stages of embryonic development. Sperm mitochondria, carrying potentially harmful paternal mtDNA, appear to be eliminated by a ubiquitin-dependent mechanism. Other accessory structures of the sperm axoneme, including fibrous sheath, microtubule doublets, outer dense fibers, and the striated columns of connecting piece, are discarded in an orderly fashion. The new methods of assisted fertilization, represented by intracytoplasmic sperm injection and round spermatid injection, bypass multiple steps of natural fertilization by introducing an intact spermatozoon or spermatogenic cell into oocyte cytoplasm.

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Consequently, the carryover of sperm accessory structures that would normally be eliminated before or during the entry of sperm into oocyte cytoplasm persist therein and may interfere with early embryonic development, thus decreasing the success rate of assisted fertilization and possibly causing severe embryonic anomalies. Similarly, foreign organelles, proteins, messenger RNAs, and mitochondrial DNAs, which may have a profound impact on the embryonic development, are propagated by the nuclear transfer of embryonic blastomeres and somatic cell nuclei. This aspect of assisted fertilization is yet to be explored by a focused effort.
KEY WORDS: Fertilization, Sperm, Spermatid, Oocyte, Embryo, Centrosome, Perinuclear theca, Intracytoplasmic sperm injection, Round spermatid injection.

I. Introduction

> ''It is conceivable, and indeed probable, that every part of the adult contains molecules derived from the male and from the female parent; and that, regarded as a mass of molecules, the entire organism may be compared to a web of which the warp is derived from the female and the woof from the male.'' (Huxley, ''Evolution in Science and Culture,'' p. 296 from Encyclopedia Britannica 1878, quoted by D. Szöllösi, 1965)

Although the role of sperm-derived structures in animal development has traditionally been overlooked and restricted to the donor of the paternal half of the future zygotic genome, new research emphasizes the importance of paternally inherited organelles and signaling molecules in fertilization and early embryogenesis. The goal of this review is to summarize our current knowledge of (i) paternally contributed zygotic structures, such as the zygotic centrosome and the male pronucleus, (ii) signaling molecules and receptors implicated in fertilization and development that are anchored to the sperm plasma membrane and perinuclear theca, and (iii) sperm accessory structures, including mitochondrial sheath and axoneme, that are discarded after fertilization. In addition, the implications of extranuclear paternal inheritance for the rapidly advancing clinical field of assisted reproduction are discussed.

II. Sperm–Oocyte Interactions

A. Requirements for Sperm Contributions

1. Haploid Genome

Most mammalian oocytes await fertilization arrested at the metaphase of second meiosis, still containing two complete sets of chromosomes entrap-

ped in the second meiotic spindle. Activation of a quiescent oocyte by the fertilizing spermatozoon results in the completion of second meiosis and extrusion of one-half of maternal chromosomes in the form of a second polar body (Schultz and Kopf, 1995), accompanied by the activation of antipolyspermy defense. Similar to oogenesis, spermatogenesis encompasses two subsequent meiotic divisions, resulting in the haploidization of the paternal genome (Clemont, 1969, 1972). The final step of spermatogenesis, spermiogenesis, then generates a set of unique sperm accessory structures designated to support sperm motility and transport through female genital tract and oocyte vestments. This process encompasses the shedding of the redundant spermatogenic cell cytoplasm containing the supernumerary organelles and transformation of the remaining organelles into sperm accessory structures (De Kretser and Kerr, 1994). Therefore, the spermatogenic cell nucleus is deprived of its nuclear skeleton, and histones in its DNA scaffold (nucleosomes) are replaced by the testis-specific DNA-binding proteins, protamines, causing the hypercondensation of the sperm chromatin into a hydrodynamic shape of the compact sperm nucleus (Oko and Clermont, 1998). Nuclear pore complexes (NPCs) and nuclear lamins become obsolete and are thus discarded at the round spermatid stage (P. Sutovsky, unpublished data). The nuclear envelope fuses with the inner face of perinuclear theca to form a compact capsule around the sperm nucleus (Courtens *et al.,* 1976; Oko and Maravei, 1995). A specialized lysosomal vesicle, the acrosome, is thought to be derived from cytoplasmic membranes of Golgi complex (Bowen, 1922; Oko and Maravei, 1995), and its exocytosis at fertilization facilitates sperm penetration through oocyte vestments (Bedford, 1968; Bleil and Wassarman, 1983). Protamines and other proteins of the sperm nucleus and accessory structures undergo post-translational modifications associated with epididymal maturation, sperm capacitation, and sperm–oocyte interactions (Bedford, 1979; Colleau *et al.,* 1997). Therefore, both oocytes and spermatozoa are equipped with one haploid set of chromosomes and a complementary arsenal of organelles and molecules necessary for fertilization. In contrast to maternal chromosomes that can decondense readily into a female pronucleus following the completion of second meiosis (Masui and Clarke, 1979), the paternal chromatin requires an additional processing step, comprising the relief of disulfide bond cross-linking and replacement of DNA-bound protamines with oocyte-derived histones, before it can participate in pronuclear development and syngamy (Krzanowska, 1982; Kopecny and Pavlok, 1975; Perreault *et al.,* 1984).

2. Activation Signal

Fertilization-competent mammalian oocytes are arrested in the metaphase of second meiosis by the action of cytostatic factor (CSF; Masui, 1982),

which is overcome by a sperm-induced signaling cascade (Schultz and Kopf, 1995). Sperm-borne factors, which are responsible for oocyte activation, may be strictly testis-specific gene products, and only the secondary messenger molecules, such as protein kinases and Ca^{2+} ions, are shared by both male and female gametes. Such a mechanism would allow for the synchronization of male and female pronuclear development while preventing the unwanted activation of oocyte cell cycle by the endogenous signaling molecules of maternal origin, yet retaining the potential for parthenogenotic initiation of development. Completion of oocyte meiosis and activation of the antipolyspermic defense, both preventing embryonic polyploidy, which is lethal to mammals, thus relay on the sperm-borne activation signal. Three basic hypotheses were formulated to explain the role of sperm in oocyte activation. The original ''conduit'' theory formulated by Jaffe (1980) assumes that sperm itself is a source of Ca^{2+} ions that induce oocyte activation. The second, ''contact'' hypothesis maintains that a receptor–ligand interaction at the level of sperm and oocyte plasma membranes triggers a signaling cascade culminating in oocyte activation (Jones and Whittingham, 1996; Swann *et al.,* 1989). Finally, the third and most recent hypothesis, also called a ''content'' model (Jones *et al.,* 1998), suggests that the fertilizing spermatozoon introduces a soluble cytosolic factor directly into oocyte cytoplasm at fertilization (Kimura *et al.,* 1998; Parrington *et al.,* 1996; Sette *et al.,* 1997). Whether it is transmitted at the level of membrane receptors or as a soluble cytosolic factor, it is generally accepted that the signal for oocyte activation is contributed by the sperm (Swann, 1996). Studies suggest that one or more such factors could be anchored in the sperm perinuclear theca (PT) (Kimura *et al.,* 1998) and transmitted into oocyte cytoplasm during its dissolution at fertilization (P. Sutovsky and R. Oko, unpublished data; see Section III,C for more details).

3. Centrosome

The ultimate goal of fertilization, i.e., the union of the maternal and paternal chromosomes into a zygotic genome, is accomplished by pronuclear apposition. This process is guided by oocyte microtubules and depends on the ability of the zygote to reconstruct the centrosome (Schatten, 1994). With few exceptions, the microtubule-based cytoplasmic motility of interphase cells, including somatic cells as well as growing oocytes and spermatogenic cells, is governed by an active centrosome composed of two cylinder-shaped centrioles surrounded by a dense halo of pericentriolar material in which the ability to enucleate microtubules resides. Such a centrosome duplicates at mitosis or meiosis to give rise to two poles of the mitotic/meiotic spindle (Gould and Borisy, 1977; Kalnins and Rogers, 1992; Schatten, 1994; Vorobjev and Chentsov, 1982). The centrosome of spermatogenic cells is initially

involved in the generation of the sperm axoneme and is later reduced to a single inactive centriole or, as evidenced in rodents, dismantled completely (Woolley and Fawcett, 1973; Manandhar *et al.,* 1998; Sutovsky *et al.,* 1999). Similarly, the proteins of pericentriolar material are rejected in the form of a cytoplasmic droplet (Manandhar *et al.,* 1998). Proportionally to the retention of one sperm centriole, which retains its ability to duplicate, animal oocytes lose both centrioles during oogenesis, but accumulate an excessive pool of pericentriolar material/centrosomal proteins (Szöllösi *et al.,* 1972; Palazzo *et al.,* 1992). At fertilization, the sperm-contributed centriole utilizes the oocyte-produced pool of pericentriolar material for its duplication and conversion into an active zygotic centrosome capable of guiding pronuclear development and formation of the first mitotic spindle (Navara *et al.,* 1994, 1996b; Sutovsky *et al.,* 1996a,b). Thus, the normal embryonic development of higher eutherians depends on the sperm-borne centriole for the organization of an active zygotic centrosome and mitotic apparatus.

4. Mitochondrial Genome?

In addition to the haploid nuclear genome, both sperm and oocytes possess an extranuclear genome represented by mitochondrial DNA (mtDNA). Mitochondria of the oocyte cytoplasm and sperm mitochondrial sheath contain up to 10 complete copies of mitochondrial genome each (Zeviani and Antozzi, 1998). Whereas the fertilizing spermatozoon brings this pool of paternal mtDNA into oocyte cytoplasm at fertilization, the inheritance of mtDNA in mammals follows a strictly maternal line (Hutchinson *et al.,* 1974; Giles *et al.,* 1980). The only feasible explanation for this paradox is that all paternally contributed mitochondria are destroyed at fertilization (Ankel-Simon and Cummins, 1994; Cummins, 1998). The coexistence of two different mitochondrial genomes in the same organism causes the condition termed heteroplasmy, which may have a detrimental effect on the embryonic development, fitness, and life span of humans and animals (Smith and Alcivar, 1993; Zeviani and Antozzi, 1997). Therefore, it appears that sperm mitochondria do not contribute to the zygotic pool of mtDNA in mammals. We are just beginning to understand the mechanism and reasons of the species-specific recognition and elimination of paternal mitochondria in mammals, yet it appears that there is an evolutionary advantage in eliminating paternal mtDNA (Boore, 1997; Hurst, 1992; Jenuth *et al.,* 1997; Marchington *et al.,* 1997). Accordingly, a variety of mechanisms evolved that prevent the sperm-borne mtDNA from either entering the oocyte or replicating in its cytoplasm (Birky, 1995). In contrast to the misconceptions about mammalian fertilization, the sperm tail is incorporated into oocyte cytoplasm and sperm mitochondria do enter oocyte cytoplasm in mammals (Ankel-Simon and Cummins, 1998). Dilution of sperm

mtDNA, oxidative damage of sperm mitochondria, and proteolytic destruction were proposed as possible mechanisms of paternal mitochondrial elimination in mammals (Cummins, 1998).

5. Sperm Tail Accessory Structures Discarded at Fertilization

In addition to the mitochondrial sheath, several other accessory structures of the sperm axoneme become obsolete after sperm incorporation into oocyte cytoplasm and are targeted for destruction. These include axonemal microtubule doublets, outer dense fibers, striated columns of the connecting piece, and the fibrous sheath of the principal piece (Hiraoka and Hirao, 1988; Shalgi *et al.,* 1994; Sutovsky *et al.,* 1996b; Szöllösi, 1965). Little is known about the mechanisms for the elimination of these structures, although it appears that they differ from the mechanisms of mtDNA destruction. It is possible that the elimination of these structures prevents their collision with normal embryonic development. Thus, the function of the sperm axoneme during fertilization is not unlike that of a rocket engine propelling the space shuttle, which is rejected and destroyed once the shuttle reaches the orbit.

B. Sperm Demembranation

1. Sperm Zona Binding, Acrosome Reaction, and Zona Penetration

The initial binding of the sperm to the egg investment, the zona pellucida (ZP), is mediated by glycoproteins on both gametes (Benoff, 1997). There appears to be a general agreement on the role of zona pellucida component ZP3 as a ZP receptor for the mouse sperm (Florman and Wassarman, 1985; Wassarman, 1990) and an inducer of sperm acrosome reaction (Bleil and Wassarman, 1983). Other zona pelucida proteins, however, may facilitate sperm–egg binding and determine the species specificity of this interaction (Rankin *et al.,* 1998). On the sperm side, several candidates were proposed for the sperm ligand that binds to ZP3 or to other zona pellucida proteins. Among others (Yanagimachi, 1994), these include proacrosin/acrosin (Jones, 1991; Urch and Patel, 1991), sperm tyrosine kinases stimulated by ZP3 (Burks *et al.,* 1986; Cheng *et al.,* 1994; Leyton and Saling, 1989), plasma membrane protein with hyaluronidase activity PH-20 (Primakoff *et al.,* 1985), SP-10 protein involved in secondary sperm binding to zona (Conrod *et al.,* 1996; Freeman *et al.,* 1994), a G-protein-coupled β-1,4-galactosyltransferase (Gong *et al.,* 1995), and phospholipase A_2 (Riffo and Parraga, 1997). The binding of ZP3 to the sperm membrane recep-

tors triggers a cascade of signaling and structural events that result in the vesiculation of the outer acrosomal membrane (Bedford, 1968; Bleil and Wassarman, 1983); dispersion of the acrosomal content, which appears to be facilitated by the acrosomal endoprotease, acrosin (Yamagata *et al.,* 1998); and secondary binding of the inner acrosomal membrane to ZP (Yanagimachi, 1994). Penetration of the sperm through zona may be a result of mechanical forces or an active enzyme-dependent digestion (Yanagimachi, 1994). It is a matter of an ongoing dispute whether the acrosomal hydrolases (Barros *et al.,* 1996) assist the penetration of zona or facilitate only the initial binding of sperm to it (Bedford, 1998). Perhaps the acrosomal matrix is an evolutionary rudiment, an idea considered by Yanagimachi (1994), and acrosomal exocytosis simply allows the secondary binding of acrosome-reacted sperm to zona via proteins anchored to the inner acrosomal membrane, such as PH-20 (Myles and Primakoff, 1997). This and other acrosomal proteins may also be helpful in the penetration of cumulus oophorus prior to zona binding (Myles and Primakoff, 1997; Bedford, 1998). Acrosomal exocytosis removes the outer acrosomal membrane and the acrosomal matrix, although the complex of inner acrosomal membrane, subacrosomal perinuclear theca, and nuclear envelope, referred to as perforatorium (Fawcett, 1975), can be seen in the sperm inside the perivitelline space and, at least in rat, enters the egg where it can persist past the first embryonic cleavage (Szöllösi, 1976). Persistence of the acrosome and/or perforatorium after assisted fertilization may affect the outcome of treatments such as the intracytoplasmic sperm injection and the round/elongated spermatid injection (reviewed in Section VII, A).

2. Sperm Oolemma Binding and Fusion

A number of sperm antigens have been implicated in the process of sperm–oolemma binding. These include the epididymal protein DE (Cohen *et al.,* 1996; Rochweger *et al.,* 1992), equatorin (Toshimori et al., 1998), vitronectin (Fusi et al., 1996), and the sperm antigens that cross-react with the fertilization-blocking antibodies MH61 (Okabe *et al.,* 1990), M29, and M37 (Saling *et al.,* 1985), and OBF 13 (Okabe *et al.,* 1988). Most of these studies were based on experiments with fertilization-blocking antibodies raised against sperm surface antigens. Although useful, such studies often do not provide sufficient information on the oolemma receptors complementary to such sperm antigens or data supporting their involvement in the fusion of sperm and oocyte plasma membranes after their initial binding. More corroborating studies were performed to demonstrate the involvement of integrins, the dimeric plasma membrane receptors for extracellular matrix proteins, in sperm–oolemma binding and fusion. Initially, Bronson and Fusi (1990) demonstrated the involvement of the integrin-anchored RGD

adhesion sequence in mammalian fertilization and the presence of integrins on the oolemma of human oocytes (Fusi *et al.,* 1992), and implicated integrins in the binding of oolemma to a specific sperm receptor. One such receptor with putative membrane fusion domain was identified in guinea pig as fertilin or PH-30 (Blobel *et al.,* 1992; Wolfsberg *et al.,* 1993). Fertilin analogs were later found in mouse (Cho *et al.,* 1997; Evans *et al.,* 1995), rat (McLaughlin *et al.,* 1997), *Cynomolgus* monkey (Ramarao *et al.,* 1995), rhesus monkey (Perry *et al.,* 1995), humans (Gupta *et al.,* 1996; Vidaeus *et al.,* 1997), rabbit (Hardy *et al.,* 1996), and bovine (Waters and White, 1994). A whole new family of fertilin-related metaloproteases with a putative viral fusion domain (ADAMs) was discovered (Wolfsberg *et al.,* 1995). Although there is a great deal of evidence supporting the role of fertilin β and integrin $\alpha 6\beta 1$ in sperm–oolemma binding (Almeida *et al.,* 1995; Evans *et al.,* 1995, 1997a,b), the evidence for their involvement in sperm–oolemma fusion is incomplete despite the presence of the viral fusion domain in the fertilin molecule. Knockout of the fertilin β gene in mice reduced, but did not completely erase, the ability of mutant sperm to bind to and fuse with the oocytes *in vitro* (Cho *et al.,* 1998). Interestingly, the knockout of fertilin completely prevented the passage of mutant sperm past the uterotubal junction and zona pellucida adhesion after the mating of wild-type females with mutant males (Cho *et al.,* 1998). Other ADAMs were implicated in sperm oolemma binding and/or fusion (Cho *et al.,* 1996; Hooft van Huijsduijnen, 1998; Yuan *et al.,* 1997). On the oocyte side, integrin $\beta 1$ function appears to be compensated by other adhesive molecules during gamete fusion in humans when suppressed by anti-integrin antibodies or RGD-containing peptides (Ji *et al.,* 1998). Such data indicate that the sperm–oolemma binding and fusion, although facilitated by fertilin–integrin binding, do not depend on it exclusively.

The fate of the sperm plasma membrane after fertilization is not known. Although some investigators suggested that the sperm plasma membrane remains as a patch on the surface of fertilized mouse and sea urchin eggs (Gabel *et al.,* 1979), it is more likely that the incorporation of the sperm plasma membrane into the oolemma occurs at fertilization (Gundersen *et al.,* 1986). Fluidity of the oolemma may cause the dispersal of sperm plasma membrane components across the oolemma (Longo, 1989; Nishioka *et al.,* 1987). Incorporation of sperm plasma membrane components into oolemma may be responsible for the oolemma-specific block to polyspermy, as demonstrated by the loss of penetrability in mouse oocytes and parthenogenotic embryos fused with fertilized eggs (Krukowska *et al.,* 1998). Another question that deserves further investigation is the regionality of sperm–oolemma binding. Yangimachi (1994) maintains that the primary binding site for oocyte microvilli on the sperm nucleus, at least in rodents, is equatorial

segment. In contrast, many of the proteins implicated in sperm–oolemma binding (see Section II,B) are scattered throughout the surface of sperm head. In bovine, we observed the binding of oocyte microvilli to the equatorial segment, as well as to the perforatorium and postacrosomal sheath of the sperm head (Sutovsky *et al.,* 1996b, 1997a,b, and unpublished data).

3. Transmission of Activation Signal into Oocyte Cytoplasm

It was postulated that the fertilizing spermatozoon may induce oocyte activation by (i) injecting free Ca^{2+} ions directly into oocyte cytoplasm as suggested in sea urchins (Jaffe, 1980), (ii) triggering a signaling cascade through a binding of a sperm plasma membrane ligand to a specific receptor on the oolemma (Jones and Whittingham, 1996; Swann *et al.,* 1989), or (iii) introducing a soluble cytosolic factor directly into oocyte cytoplasm (Kimura *et al.,* 1998; Parrington *et al.,* 1996; Sette *et al.,* 1997). Substantial evidence supports each of these hypotheses, which, however, may be relevant only to certain animal species or taxa (Schultz and Kopf, 1995). For example, evidence for the signaling pathway triggered by fertilin–integrin binding was generated in amphibians (Iwao and Fujimura, 1996; Shilling *et al.,* 1998), but no such observation was made in mammals. Recent studies seem to favor the cytosolic factor hypothesis over the Ca^{2+} bomb and receptor signaling theory (Jones *et al.,* 1998; Parrington *et al.,* 1996). It is likely that such an oscillogenic factor(s) or oscillogen(s) is anchored in the sperm perinuclear theca (Kimura *et al.,* 1998; P. Sutovsky and R. Oko, unpublished data; see Section III,C). Such data are based on ultrastructural observations and on the microinjections of crude sperm head extracts or purified PT extracts into oocyte cytoplasm. Kimura *et al.,* (1998) proposed that the cytoskeletal component of PT, calicin, could have oscillogenic activity, although there are no data supporting this idea by revealing a known signaling domain in the amino acid sequence of this protein. Although previous studies showed that PT is removed from the surface of the sperm nucleus during sperm incorporation (Sutovsky *et al.,* 1997a; Fig. 1), new data demonstrate that it later becomes incorporated into oocyte cytoplasm and dissolves concomitantly with the progression of the pronuclear development (P. Sutovsky and R. Oko, unpublished data). Such an observation may explain the transmission of the sperm-anchored oscillogens into oocyte cytoplasm without the need for large ''pores'' in the oolemma, the existence of which was proposed by Jones *et al.,* (1998). The dissolution of PT, sufficient to induce oocyte activation, can be seen in a small region of sperm head that fuses with the oolemma when the sperm entry is prevented either by cytochalasin B treatment (Sutovsky *et al.,* 1996b) or by the natural block of polyspermy (P. Sutovsky and R. Oko, unpublished data).

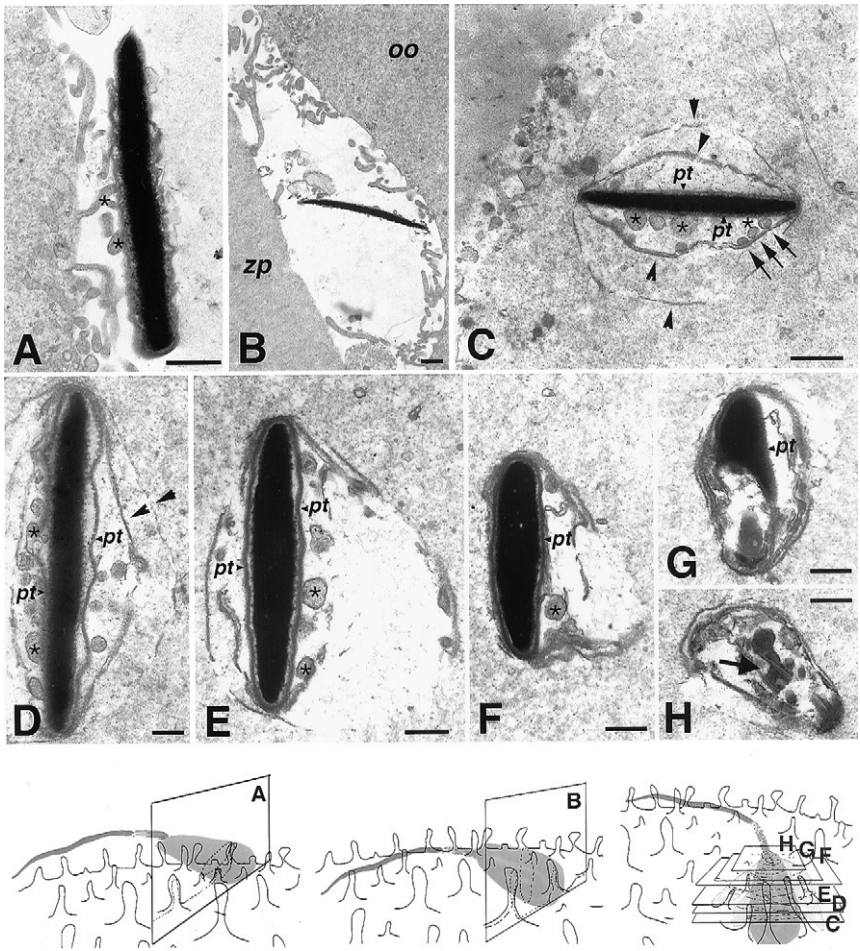

FIG. 1 Sperm incorporation and removal of the perinuclear theca (pt) in *in vitro*-fertilized bovine oocytes, as seen by transmission electron microscopy. Schematic drawings below the micrographs show the sectioning plane for each of the above images. Bull sperm initially bind flat to the tips of oocyte microvilli (A; asterisks), rotate for 90° along their longitudinal axes (B; zp, zona pellucida; oo, oocyte cytoplasm), and enter the oocyte cytoplasm perpendicularly to the oocyte surface (C–H; serial sections of the same sperm head). The sperm vestments, including plasma membrane and inner acrosomal membrane (C–F; arrowheads), separate from the sperm nucleus and the oocyte microvilli (asterisks) become intercalated between them and PT. Note the binding of oocyte microvilli to subacrosomal PT in C–F and to the inner aspect of the equatorial segment in C (arrows). The sperm centriole (arrow) in H is still intact inside the structures of the connecting piece. Scale bars: 500 nm (A–C) or 200 nm (D–H). Reprinted with permission from Sutovsky *et al.* (1997).

C. Removal of Sperm Perinuclear Theca

1. Morphogenesis of Perinuclear Theca during Spermatogenesis

Perinuclear theca, also called perinuclear substance or perinuclear matrix (Bellvé *et al.,* 1992; Courtens *et al.,* 1976; Lalli and Clermont, 1981; Oko and Clermont, 1988), is a cytoskeletal coat of the sperm nucleus composed of myelin and cytokeratin-like proteins. PT is inserted between the sperm plasma membrane and nuclear envelope, with which it appears to be intimately associated and at least in part fused (Oko and Maravei, 1995). The PT proteins first appear on the apical nuclear surface at the round spermatid stage of spermatogenesis (Oko, 1995; Oko and Maravei, 1995). Using the classification of spermiogenesis by Berndston and Desjardins (1974) and Barth and Oko (1989), Oko and Maravei (1995) presented a detailed description of PT biogenesis in bull. Initially, PT proteins accumulate on the surface of the Golgi-derived acrosomal granule, which is not attached to the nuclear surface in step 1 and step 2 round spermatids. Formation of the acrosomal cap is parallelled by the attachment of the acrosomal granule to the nuclear envelope/PT complex and by the extension of PT over the apical surface of a step 4 round spermatid, resulting in the formation of a continuous subacrosomal PT layer. As the spermatid nucleus condenses and elongates, so does subacrosomal PT. In addition to the subacrosomal layer separating the acrosome from the nuclear envelope, the equatorial segment of the acrosome becomes covered by PT on its surface in step 8 spermatid. In a fully differentiated spermatozoon, the postacrosomal sheath of PT extends to the implantation fossa on the posterior nuclear surface and the acrosome becomes sandwiched between the subacrosomal and outer periacrosomal layer of PT, effectively covering the whole sperm nucleus at this stage of spermiogenesis.

Formation of the PT at the round spermatid stage is preceded by the transcription of numerous PT proteins (summarized in Table I). In rat, a major PT protein PERF15, a perforatorium protein of 15 kDa (Oko and Morales, 1994), begins to be transcribed in the meiotic phase of spermatogenesis and its expression culminates in postmeiotic round spermatids, at this time being coordinated with its translational activity. The cessation of this transcriptional and translational activity is seen during spermatid elongation. This pattern of developmental expression was also shown for other rat PT proteins and for the major proteins of the bull sperm PT, including a 15.5-kDa band not homologous to rat PT 15 and the other bands of 25, 28, 32, 36, and 60 kDa (Oko and Maravei, 1994, 1995). Biogenesis of PT appears to be a prerequisite for normal shaping of the sperm nucleus, as the lack of PT in humans coincides with an aberrant nuclear shape in

TABLE I
Components of Mammalian Sperm Perinuclear Theca

Protein	M_r (kDa)	Species	Reference
Calicin	58–60	Bull, mice, human, boar, hamster, rat guinea pig	Longo *et al.* (1987); Olson and Winfrey (1988); Paranko *et al.* (1988)
Cylicin	58	Bull, human	Hess *et al.* (1993)
Multiple band proteins	56–74	Bull	Longo and Cook (1991)
PERF15	15	Rat, mouse	Oko and Morales (1994); Korley *et al.* (1997)
PT15	15.5	Bull	Oko and Maravei (1995)
Stat4	87	Mouse	Herrada and Wolgemuth (1997)
—	25	Bull	Oko and Maravei (1995)
—	28	Bull	Oko and Maravei (1995)
—	32	Bull	Oko and Maravei (1995)
—	36	Bull	Oko and Maravei (1995)
—	48–50	Mouse	Bellvé *et al.* (1990, 1992)

spermatozoa of patients suffering from globozoospermy (Escalier, 1990). It is possible that an orchestrated interaction of PT with the microtubule-based spermatid manchette is required for the completion of this process (Longo and Cook, 1991).

2. Removal of Perinuclear Theca at Fertilization and Its Possible Role in Sperm–Oolemma Binding

Although the aforementioned data emphasize its importance for normal spermiogenesis, the compact and sturdy PT would become an unsurpassable barrier at fertilization, when it would prevent the remodeling of the sperm nucleus into a male pronucleus. The sperm nucleus is transformed into a male pronucleus by the release of sperm nuclear protamines followed by the uptake of histones and other molecules from oocyte cytoplasm (Ecklund and Levine, 1975; Kopecny and Pavlok, 1975; Perreault *et al.,* 1984). This would be impossible without the elimination of sperm PT (Sutovsky *et al.,* 1997a) and without the *de novo* formation of the nuclear envelope (NE) with functional nuclear pore complexes (Sutovsky *et al.,* 1998b). Data in mouse (Usui, 1996; Usui *et al.,* 1997) and bovine (Sutovsky *et al.,* 1997a) suggest that sperm PT is removed from the surface of the sperm nucleus at an initial stage of incorporation and that sperm chromatin enters oocyte cytoplasm completely devoid of all membranes, including its intrinsic nuclear envelope (Sutovsky *et al.,* 1998b; Usui and Yanagimachi, 1976; Usui

et al., 1997). In rat, the whole perforatorium is detached from the sperm nucleus inside the oocyte cytoplasm and sometimes can be traced throughout the pronuclear development (Szöllösi, 1976). In bovine (Fig. 1), oocyte microvilli bind to sperm PT after the fusion of sperm plasma membrane and oolemma and become intercalated between individual sheaths of PT. Subsequently, PT is removed and the sperm nucleus is exposed to oocyte cytoplasm. The persistence of sperm PT on the surface of intact bull spermatozoa injected into the cytoplasm of mature bovine oocytes causes the failure of pronuclear development (Sutovsky *et al.,* 1997a). Similarly, the aberrant pronuclear development associated with the persistence of acrosome and/or subacrosomal PT was reported after intracytoplasmic sperm injection in humans (Sathananthan *et al.,* 1997a; Bourgain *et al.,* 1998) and rhesus monkeys (Hewitson *et al.,* 1996, 1999; Sutovsky *et al.,* 1996a). These data suggest that the removal of sperm PT is a prerequisite for normal pronuclear development in mammals.

Integrin–fertilin binding (Almeida *et al.,* 1995; Evans *et al.,* 1995; see Section II,B) seems to play an important, but not exclusive, role in sperm–oolemma binding. Fertilin knockout, that failed to abolish fertility in mice completely (Cho *et al.,* 1998), suggests that other receptors for oolemma may exist in mammalian spermatozoa. With regard to the evidence of oocyte microvilli binding to sperm PT (Sutovsky *et al.,* 1997a), it is tempting to speculate that one or more PT proteins may serve as a secondary sperm receptor(s) for oolemma. Acrosomal exocytosis exposes the inner acrosomal membrane of the perforatorium that is tightly bound to and perhaps fused with the subacrosomal PT (Oko and Maravei, 1994). In addition, the surface of the equatorial segment, which appears to be a preferred site for the attachment of oocyte microvilli at fertilization (Yanagimachi, 1994; Bedford and Cooper, 1978), is underlined by the outer leaf of PT. Besides lending structural stability to the inner acrosomal membrane, PT proteins may protrude on the surface of perforatorium or equatorial segments and interact with the specific receptor(s) on the oolemma during fertilization. Once the oolemma fuses with sperm plasma membrane, the resulting ''zygotic'' plasma membrane becomes connected to the PT. Exposure of the sperm PT by the removal of sperm plasma membrane with permeabilizing agents such as Triton X-100 and lysophosphatidilcholine increases the binding of such permeabilized spermatozoa to zona-free oocytes (Sutovsky *et al.,* 1997b).

3. Perinuclear Theca Internalization for Oocyte Activation and Other Factors Present in Sperm Perinuclear Theca

The role of PT during natural fertilization may not be restricted to that of a barrier to be removed. Evidence is accumulating that PT contains a

factor(s) responsible for the initiation of fertilization-specific calcium oscillations, which trigger oocyte activation. The intracytoplasmic injection of sperm from patients suffering from globozoospermia, a spermatogenic disorder associated with the absence of PT (Bacetti *et al.,* 1997; Escalier, 1990; Holstein *et al.,* 1973a,b), fails to activate donor human oocytes and to induce fertilization-specific calcium oscillations (Battaglia *et al.,* 1997; Rybouchkin *et al.,* 1996). Kimura *et al.* (1998) showed that oocyte activation and normal embryonic development can be induced by the intracytoplasmic injection of crude PT extracts in mice. The major candidate for sperm oscillogenic protein, oscillin or glucosamine-6-phosphate deaminase (Parrington *et al.,* 1996), was found in the region of the equatorial segment of the sperm head, which is enveloped by PT sheaths, yet the recombinant protein failed to induce oocyte activation after microinjection (Wolosker *et al.,* 1998). Although no PT remnants are found in the perivitelline space of fertilized oocytes and on the surface of mammalian sperm nuclei incorporated into oocyte cytoplasm during natural fertilization (Sutovsky *et al.,* 1996b; Sutovsky and Schatten, 1997), fragments of PT can be detected in the oocyte cytoplasm near the male PN at an early stage of pronuclear development (P. Sutovsky and R. Oko, unpublished data). Similarly, PT remnants are not seen in the male pronuclei of rhesus monkey oocytes fertilized *in vitro* (Sutovsky *et al.,* 1996a). It is likely that the oscillogenic factors are released from PT as it dissolves in the oocyte cytoplasm. Other factors important for the success of fertilization and preimplantation embryonic development may be anchored to sperm PT and brought with it into oocyte cytoplasm at fertilization, as evidenced by the finding of transcription factor Stat4 in the perinuclear theca of the mouse spermatozoa (Herrada and Wolgemuth, 1997)

III. The Centrosome, Sperm Nucleus, and Pronucleus

A. Centrosomal Inheritance, Pronuclear Apposition, and Microtubule Organization

1. Centrosome Reduction during Gametogenesis

The functional centrosome is composed of two centrioles, the pinwheel-like cylinders containing nine triplets of microtubules and a relatively homogeneous pericentriolar material around them (Kalnins and Rogers, 1992; Schatten, 1994; Vorobjev and Chentsov, 1982). Although the centrioles may provide structural stability and serve as a blueprint for the assembly and duplication of an active centrosome, it is the pericentriolar material

that possesses the microtubule-nucleating activity (Gould and Borisy, 1977). The polymerization of tubulin starts from the ring-like structures in pericentriolar material, which contain a unique tubulin species, γ-tubulin (Joshi, 1993; Moritz *et al.,* 1995; Raff, 1996; Stearns *et al.,* 1991; Zheng *et al.,* 1995). Other centrosomal proteins, including centrin (Sanders and Salisbury, 1989), centrosomin (Joswig *et al.,* 1991), katanin (McNally *et al.,* 1996), MPM-2 complex (Westendorf *et al.,* 1994), ninein (Bouckson-Castaign *et al.,* 1996), and pericentrin (Doxey *et al.,* 1994), were localized in the pericentriolar material of various organisms. In contrast to this general model of the centrosome, mammalian oocytes lose centrioles during oogenesis and their meiotic spindle poles are occupied by the loosely assembled, acentriolar microtubule-organizing centers (MTOCs; Hertig and Adams, 1967; Sathananthan *et al.,* 1997; Szöllösi *et al.,* 1972; Zamboni and Mastroianni, 1966). In a complementary fashion, the centrosome of the mammalian spermatozoa is reduced to a single centriole or is eliminated completely during spermiogenesis (Sutovsky *et al.,* 1999). Two centrioles are still seen at the base of the sperm tail in mammalian round spermatids (Fouquet *et al.* 1998; Manandhar *et al.,* 1998). The proximal centriole is attached to the basal plate of the sperm axoneme in the implantation fossa, which in turn serves for the attachment of the axoneme to the nucleus. The distal centriole is oriented perpendicularly to the proximal one and seems to serve as a mold for the assembly of axonemal microtubule doublets that grow directly from the distal ends of its triplets. In most eutherian mammals, as well as in marsupials (Table II), the proximal centriole is retained during spermiogenesis and spermiation. In a mature spermatozoon (Fig. 2), the proximal centriole is embedded in a dense mass of capitulum that probably replaces the active pericentriolar material and is inert with regard to microtubule nucleation. The distal centriole is dismantled during spermatid elongation, and the distal centriolar vault of a fully differentiated spermatozoon is empty (Fouquet *et al.,* 1998; Manandhar *et al.,* 1998). In rodents, represented by mouse (Manandhar *et al.,* 1998), hamster (Fouquet *et al.,* 1998), guinea pig (Fouquet *et al.,* 1998), and rat (Woolley and Fawcett, 1973), both centrioles disappear during spermiogenesis. Manandhar *et al.,* (1998) divided the elimination of the sperm centrosome and centrioles in mouse into three steps including (i) loss of microtubule-nucleating activity, (ii) disappearance of centrosomal proteins such as γ-tubulin and centrin, and (iii) disintegration of both centrioles. Therefore, the centrosomal reduction during male and female gametogenesis occurs in an unequal, yet complementary fashion. Whereas oocytes lose both centrioles and retain active pericentriolar material, only one of the two centrioles is lost in the spermatozoa. The reduced sperm centrosome lacks active pericentriolar material capable of microtubule nucleation. As an exception to this rule, rodent

TABLE II

Number of Centrioles in Mature Spermatozoa of Mammals Studied to Date and Their Implication in the Organization of Pronuclear Apposition and First Mitosis[a]

Species	No. of centrioles in sperm	No. of centrioles in oocyte	Sperm aster	Sperm centriole shown in zygotic MTOCs	Sperm centriole duplication shown	No. of centrioles per pole in first mitotic spindle	Reference[b]
Human	1	0	Yes	Yes	Yes	1–2	16, 17, 21
Rhesus	1	0	Yes	Yes	Yes	?	5, 21
Bovine	1	0	Yes	Yes	Yes	?	9, 12, 13, 22, 23
Sheep	1	0	Yes	Yes	Yes	1–2	3, 8
Pig	1	?	Yes	No	No	?	7, 25
Rabbit	1	?	Yes	No	No	?	10, 14
Hamster	0	?	No	No	No	?	4, 6
Mouse	0	0	No	No	No	?	4, 11, 17–19, 24
Rat	0	?	?	No	No	?	4, 26
Guinea Pig	0	?	?	No	No	?	4
Monodelphis	1	?	Yes	No	No	?	1, 2

[a] Adapted from Sutovsky *et al.* (1999).

[b] (1) Breed *et al.,* 1996; (2) Breed and Sutovsky, unpublished data; (3) Crozet, 1990; (4) Fouquet *et al.,* 1998; (5) Hewitson *et al.,* 1996; (6) Hewitson *et al.,* 1997; (7) Kim *et al.,* 1996; (8) Le Guen and Crozet, 1989; (9) Long *et al.,* 1993; (10) Longo, 1976; (11) Manandhar *et al.,* 1998; (12) Navara *et al.,* 1994; (13) Navara *et al.,* 1996a; (14) Pinto-Correia *et al.,* 1994; (15) Sathananthan *et al.,* 1991; (16) Sathananthan *et al.,* 1996; (17) Schatten *et al.,* 1985b; (18) Schatten *et al.,* 1986; (19) Schatten *et al.,* 1991; (20) Simerly *et al.,* 1995; (21) Sutovsky *et al.,* 1996a; (22) Sutovsky *et al.,* 1996b; (23) Sutovsky and Schatten, 1997; (24) Szöllösi *et al.,* 1972; (25) Szöllösi and Hunter, 1973; (26) Woolley and Fawcett, 1973.

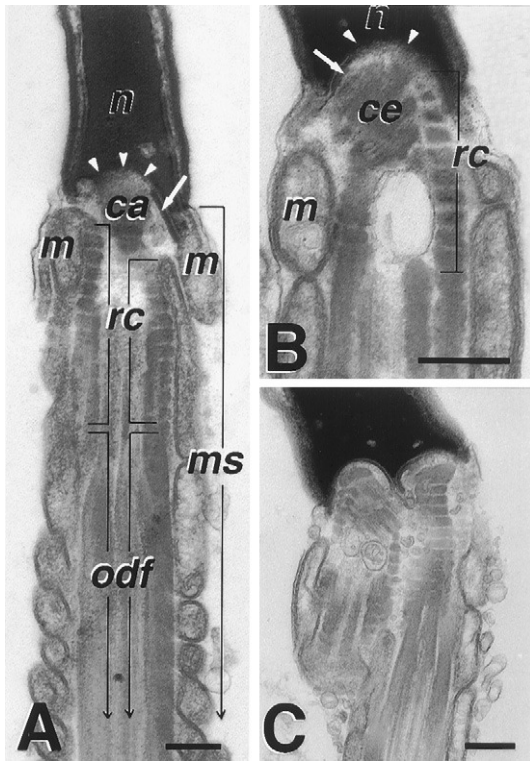

FIG. 2 Ultrastructure of the connecting piece of the bull spermatozoon (A, B). The centriole (ce) is embedded in the dense mass of the sperm capitulum (ca) caged by nine ribbed columns (rc; only two or three of them can be seen on these cross sections), which are the continuation of the nine outer dense fibers (odf) of the sperm midpiece/principal piece. The whole connecting piece is anchored to the implantation fossa (arrowheads) of the sperm nucleus (n) by the basal plate (arrows) and wrapped in the uppermost flat mitochondria (m) of the mitochondrial sheath (ms). (C) An aberrant bull sperm with two implantation fossae and two tails. Bars: 500 nm. Reprinted with permission from Sutovsky *et al.* (1996b).

spermatozoa are deprived of both centrioles, thus displaying a complete lack of centrosome.

2. Centrosome Reconstitution at Fertilization

In contrast to the relatively equal nuclear genomic contributions by both parents, the cytoplasmic inheritance in mammals varies substantially for individual organelles. As early as 1901, Theodore Boveri formulated his theory of paternal centrosome inheritance, later supported by the absence

of centrioles reported in the oocytes of mouse (Szöllösi *et al.,* 1972), rabbit (Zamboni and Mastroianni, 1966), bovine (Sathananthan *et al.,* 1997b), and human (Hertig and Adam, 1967) and by the presence of an intact proximal centriole in the spermatozoa of most animal species (Table II). The reconstitution of the mammalian zygotic centrosome was described in detail by ultrastructural and immunocytochemical studies in bovine (Long *et al.,* 1993; Navara *et al.,* 1994, 1996a; Sutovsky *et al.,* 1996b; Sutovsky and Schatten, 1997), humans (Asch *et al.,* 1995; Sathananthan *et al.,* 1991, 1996; Simerly *et al.,* 1995), pig (Kim *et al.,* 1996; Szöllösi and Hunter, 1973), rabbit (Longo, 1976; Pinto-Correia *et al.,* 1994), rhesus monkey (Hewitson *et al.,* 1996; Sutovsky *et al.,* 1996a; Wu *et al.,* 1996), and sheep (Crozet, 1990; Le Guen and Crozet, 1989. Ultrastructural studies in bovine (Sutovsky *et al.,* 1996b; Sutovsky and Schatten, 1997) and rhesus monkey (Sutovsky *et al.,* 1996a) provide a detailed account of cellular events at fertilization that includes the assembly of an active zygotic centrosome. In the first step, the complex structure of the sperm tail connecting piece (Fig. 2), in which the sperm centriole is caged, must be disassembled in order to release the proximal centriole into the oocyte cytoplasm (Fig. 3). Initially, proximal mitochondria of the sperm mitochondrial sheath are removed from the surface of the connecting piece. This step is followed by the breakdown of nine striated columns that are the continuation of axonemal outer dense fibers. This event unmasks the proximal centriole and allows for a free access of oocyte cytoplasm to it. It appears that the sperm centriole remains loosely associated with at least one of the striated columns (Fig. 3), which explains why the sperm tail remains in the vicinity of the male pronucleus and mitotic spindle throughout pronuclear and early embryonic development (Sutovsky *et al.,* 1996b).

Following its exposure to oocyte cytoplasm, the proximal centriole begins to attract pericentriolar material from it. The presence of γ-tubulin and likely other centrosomal proteins in pericentriolar material then enables the enucleation of microtubules into a radial array called the sperm aster. Sperm aster microtubules provide tracks for the apposition of the male and female pronuclei (Schatten, 1994) and may facilitate the recruitment of preassembled nuclear pore complexes and other organelles and molecules to the developing pronuclear compartment (Sutovsky *et al.,* 1998). The original sperm centriole replicates prior to the first mitosis, during which it can be found at one of the spindle poles (Sathananthan *et al.,* 1996). The daughter centriole arising from this replication travels toward the opposite side of apposed pronuclei, where it organizes the second spindle pole (Sathananthan *et al.,* 1996; Sutovsky *et al.,* 1996a,b). Instead of being of exclusively paternal origin, the zygotic centrosome of nonrodent mammals thus appears to be a blend of the paternally contributed centriole

complex and maternally derived pericentriolar material, which should therefore be referred to as biparentally inherited organelle.

3. Maternal Inheritance of Centrosome in Rodents and Other Exceptions of the Centrosomal Rule

As mentioned earlier, rodent spermatozoa lose both centrioles during spermiation and as such cannot contribute to the formation of the zygotic centrosome. This intriguing discovery is corroborated by studies of mouse fertilization showing that instead of forming a single sperm aster, multiple cytoplasmic asters of maternal origin occupy the cytoplasm of fertilized oocytes and provide the motile force for pronuclear apposition (Schatten *et al.,* 1985b, 1986). In hamster, cytasters are formed during natural fertilization, and human spermatozoa injected into hamster oocytes do not develop a sperm aster (Hewitson *et al.,* 1997). In contrast to other eutherians and even marsupials, the biogenesis of zygotic centrosome in rodents is under strictly maternal control. Although the reasons for such evolutionary diversification of centrosomal inheritance in mammals are not known, the rudimentary mechanism of maternal centrosomal control may have been retained in nonrodent mammals, as well as in rodents. Parthenogenetic activation of cow (Navara *et al.,* 1994), pig (Kim *et al.,* 1996), rhesus monkey (Wu *et al.,* 1996), and rabbit (Pinto-Correia *et al.,* 1993; Szöllösi and Ozil, 1991) oocytes is followed by seemingly normal mitosis and embryonic development up to the blastocyst stage. Centrioles in rabbit parthenogenotes are not reformed until the blastocyst stage (Szöllösi and Ozil, 1991), which does not preclude the occurrence of mutliple rounds of embryonic cleavage therein. Although the parthenogenetic embryos die before reaching organogenesis, this may not be due to the absence of paternal centrosomal components. Experiments with the microinjection of round spermatids into the oocyte cytoplasm of species with biparentally contributed zygotic centrosomes suggest that the centrosomal activity of a sperm centrioleturned zygotic centrosome can be replaced by maternally controlled microtubule assembly and thus may be a preferred, but not an exclusive, mode of centrosomal inheritance in mammals (see Section VII,B). This view is also supported by the first successful cloning experiments in which the electrofusion with a somatic cell presumably induced the formation of a functional mitotic apparatus in reconstituted nonrodent embryos (Campbell *et al.,* 1996; Cibelli *et al.,* 1998; Kato *et al.,* 1998; Vignon *et al.,* 1998).

4. Requirements for Centrosomal Activity throughout Fertilization and Pronuclear Development

Whereas microfilament- and intermediate filament-based cytoskeletal networks exist in mammalian oocytes, the crucial role of organelle motility in

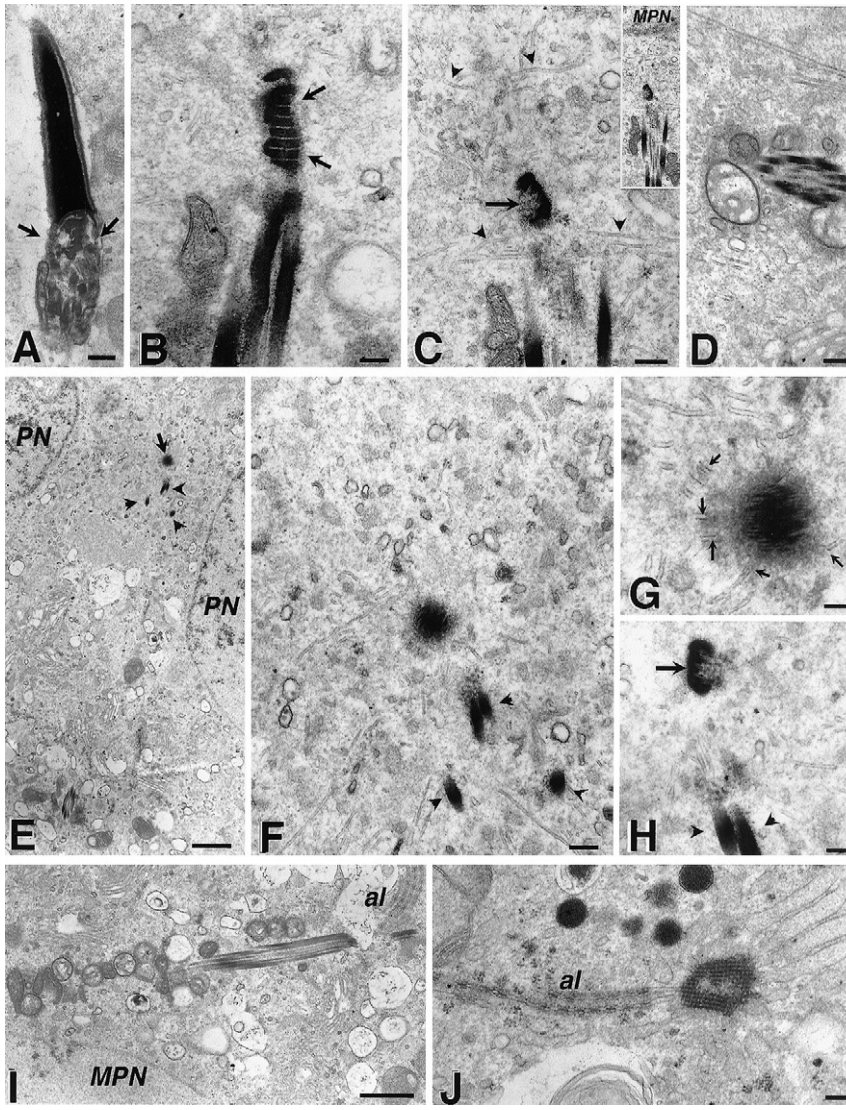

FIG. 3 Disassembly of the connecting piece and formation of the sperm aster in bovine zygotes. (A) An early stage of sperm incorporation; note the absence of sperm mitochondria from the connecting piece (arrows). Serial sections of the sperm connecting piece (B, C) demonstrate the attachment of the sperm centriole (C; arrow) to the residual ribbed column (B; arrows). Abundant microtubules (arrowheads) suggest that this centriole is an organizing center of the sperm aster. (C, inset) Attachment of the sperm axoneme to the male pronucleus (MPN). Although mitochondria are absent from the connecting piece in B and C, D shows the loose association of the mitochondrial sheath with the midpiece. (E) Position of the centrosome (arrow) during pronuclear (PN) apposition. A higher magnification of this image

the zygote has been attributed to microtubules. Microtubules provide the track for pronuclear apposition, most likely through the minus end-oriented microtubule motor proteins (Reinsch and Gonczy, 1998). Movement of pronuclei on the microtubule tracks can be mimicked in a cell-free system by mixing frog egg extracts with synthetic nuclei composed of DNA-coated microspheres (Reinsch and Karsenti, 1997). Consequently, the treatment of fertilized oocytes with microtubule disruptors such as nocodazole (Navara *et al.,* 1994; Sutovsky *et al.,* 1996b) prevents pronuclear apposition and development. Besides pronuclear motility, sperm aster microtubules may play an important role in pronuclear development. Enlargement of pronuclei and building of the nuclear skeleton capable of supporting DNA replication are complex processes requiring a continuous influx of molecules from the cytoplasm (Poccia and Collas, 1996). As reviewed in Section V, pronuclear development also requires the reconstitution of a continuous nuclear envelope from cytoplasmic membrane vesicles, which also encompasses the formation of nuclear lamina and functional nuclear pore complexes. Further pronuclear enlargement requires the recruitment of more membrane vesicles into the pronuclear region of zygotic cytoplasm, which is likely facilitated by the sperm aster microtubules. In a similar fashion, nuclear pore complexes, which are preassembled into cytoplasmic annulate lamellae on oocyte activation (Sutovsky *et al.,* 1998b), appear to be transported toward the developing pronuclei along the microtubule tracks. Disruption of the sperm aster with nocodazole indeed prevents the insertion of nuclear pores into the NE and pronuclear development, as well as pronuclear apposition (Sutovsky *et al.,* 1998b). Annulate lamellae, which are stacks of endoplasmic reticulum-derived membrane vesicles perforated by nuclear pore complexes, thus may serve as a vehicle for the rapid movement of preassembled nuclear pore complexes toward the developing pronuclei. Cytoplasmic movement of other organelles, including Golgi, ER, and mitochondria (Barnett *et al.,* 1996; Sciaky *et al.,* 1997; Terasaki *et al.,* 1996), may be governed by microtubule-associated motor proteins such as kinesin and dynein. This may also be true for species with the exclusively maternal mode of centrosomal inheritance such as hamster, in which the rearrangement of mitochondria and cytoplasmic intermediate-filament sheaths at fertilization

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(F, G) shows the minus ends of the sperm aster microtubules embedded in the dense mass of pericentriolar material (arrows). The distal end of this centriole (H; serial section) is still attached to the residual capitulum (arrow). Arrowheads in E, F, and H point to the cross sections of the outer dense fibers. Stacks of annulate lamellae (al) are associated with the sperm axoneme at the male PN (MPN) in H. (I) Formation of the annulate lamellae (al) in the cytoplasm of fertilized egg. Bars: 100 nm (G), 200 nm (B, D, F, H), 300 nm (C), 500 nm (A), and 1 μm (E, I). Reprinted with permission from Sutovsky *et al.* (1996).

depends on microtubules (Barnett *et al.,* 1996; J. Squirrell and P. Sutovsky, unpublished data). Another interesting question is whether the sperm aster microtubules can facilitate the influx of cytosolic proteins into the pronuclei. Relocation of NuMA (Compton and Cleveland, 1994; Merdes and Cleveland, 1998), a spindle pole protein associated with early sperm aster, to the nuclear skeleton where it resides during pronuclear development does not require microtubules (Navara *et al.,* 1996c). Centrosomal proteins such as centrin and γ-tubulin also appear to be recruited from cytosol without the active participation of microtubules (Navara *et al.,* 1995). In contrast, the recruitment of nuclear lamins to the pronuclei, pronuclear development, and DNA replication in mouse seem to depend on microtubule assembly (Schatten *et al.,* 1989).

5. Factors Affecting the Assembly of Zygotic Centrosome

What factors are necessary for the reconstitution of mammalian zygotic centrosome? First, it appears that the disassembly of the sperm tail connecting piece, required for the release of the sperm proximal centriole into oocyte cytoplasm, depends on the reduction of disulfide bond cross-linking in the sperm mitochondria, striated columns, and capitulum. Depletion of the endogenous reducing substance of oocyte, tripeptide glutathione (γ-glutamylcysteinel-glycine; Meister and Anderson, 1983), with the specific inhibitor of its synthesis, buthionine sulfoximine (BSO; Griffith and Meister, 1979), reduces the rates of sperm aster formation and pronuclear apposition in bovine zygotes by preventing the release of proximal centriole from the connecting piece (Sutovsky and Schatten, 1997). The effect of disulfide bond reduction on the integrity of the sperm tail connecting piece can be mimicked by treatment with reducing agents such as dithiothreitol (DTT) (Sutovsky *et al.,* 1997c). Such treatment is also necessary for the reconstitution of mammalian centrosome in a cell-free system composed of *Xenopus* egg extracts and permeabilized mammalian spermatozoa (Navara *et al.,* 1995). In contrast to mammalian sperm, disulfide bond-reducing treatment is not necessary for the sperm aster reconstitution from homologous frog sperm featuring a relatively simple structure of sperm tail and a lack of disulfide bond stabilization (Felix *et al.,* 1994; Stearns and Kirschner, 1994). Other factors are likely to contribute to the release of the sperm-borne centriole from the connecting piece into the zygotic cytoplasm. Striated columns contain an MPM-2 antibody-reactive 84-kDa phosphoprotein (Long *et al.,* 1997; Schalles *et al.,* 1998) and possibly other protein kinase substrates. Accordingly, the phosphorylation and dephosphorylation of the connecting piece have been implicated in the sperm aster formation in rabbit (Pinto-Correia *et al.,* 1994) and bull (Long *et al.,* 1997). The release of Ca^{2+} ions into oocyte cytosol at fertilization may affect the conformation

and activity of some proteins in the connecting piece of the fertilizing spermatozoon. Centrin, a Ca^{2+}-binding protein with a proven role in protozoan flagellar excision (Sanders and Salisbury, 1989), is present in the connecting piece of mammalian spermatozoa (Navara *et al.,* 1995; Zoran *et al.,* 1994) and was implicated in the excision of the sperm tail from the nuclear implantation fossa and in the regulation of sperm aster assembly (Zoran *et al.,* 1994; Fechter *et al.,* 1995).

The release of the sperm centriole into oocyte cytoplasm is followed by the assembly of pericentriolar material. γ-tubulin, the ring-like polymer of which serves as a blueprint for the assembly of microtubule protofilaments from α- and β-tubulin dimers, is recruited into the sperm centriole at the onset of sperm aster formation in nonrodent mammals and frogs (Felix *et al.,* 1994; Navara *et al.,* 1995; Steams and Kirschner, 1994; Sutovsky *et al.,* 1996b). Similarly, γ-tubulin was found in the acentriolar MTOCs of mouse zygotes (Palacios *et al.,* 1993). A multiprotein complex containing γ-tubulin and at least six other proteins appears to be involved in the nucleation of microtubules (Zheng *et al.,* 1995).

The original sperm centriole duplicates inside the zygotic centrosome and the whole centrosome itself duplicates as the daughter centriole begins its migration toward the site of the future spindle pole (Sathananthan *et al.,* 1996; Sutovsky *et al.,* 1996a,b). In somatic cells, the duplication of centrioles appears to be under control of the cell cycle via the p53 tumor suppressor (Fukasawa *et al.,* 1996; Cross *et al.,* 1995). Centrioles at the poles of the first mitotic spindle inside the fertilized human oocytes duplicate one more time before the onset of first mitosis (Sathananthan *et al.,* 1996), which means that a single sperm centriole gives rise to three new daughter centrioles during a single round of the cell cycle.

B. Remodeling of Sperm Nucleus and Pronuclear Development

1. Disulfide Bond Reduction and Unmasking of Sperm DNA

Nuclei of mature mammalian spermatozoa contain the hypercondensed chromatin packaged by the DNA-binding proteins protamines. Protamines and possibly other cysteine-rich sperm proteins become cross-linked by disulfide bonds during epididymal storage (Bedford, 1979; Calvin and Bedford, 1971; Bedford and Calvin, 1974). Because the protamines do not support the three-dimensional structure allowing DNA transcription and replication, they have to be replaced by oocyte-derived histones at fertilization (Ecklund and Levine, 1975; Krzanowska, 1982; Kopecny and Pavlok, 1975; Perreault *et al.,* 1984, 1988). This event is accompanied by the loosen-

ing of the nuclear structure and by the swelling of the sperm nucleus, and depends on the disulfide bond-reducing activity of the oocyte-produced tripeptide glutathione (GSH; Meister and Anderson, 1983; Perreault, 1984, 1990; Sutovsky and Schatten, 1997). GSH also seems to facilitate the disassembly of the sperm tail connecting piece that houses the sperm centriole (Sutovsky and Schatten, 1997) and to support the formation of the first mitotic spindle (Zuelke *et al.,* 1997). Such a mechanism of oocyte-induced sperm decondensation is exclusive to mammals, as other vertebrate taxa do not possess the ability to stabilize sperm accessory structures during sperm storage (Bedford, 1979). Nucleoplasmin was implicated in the process of sperm decondensation in amphibians (Philpott *et al.,* 1991).

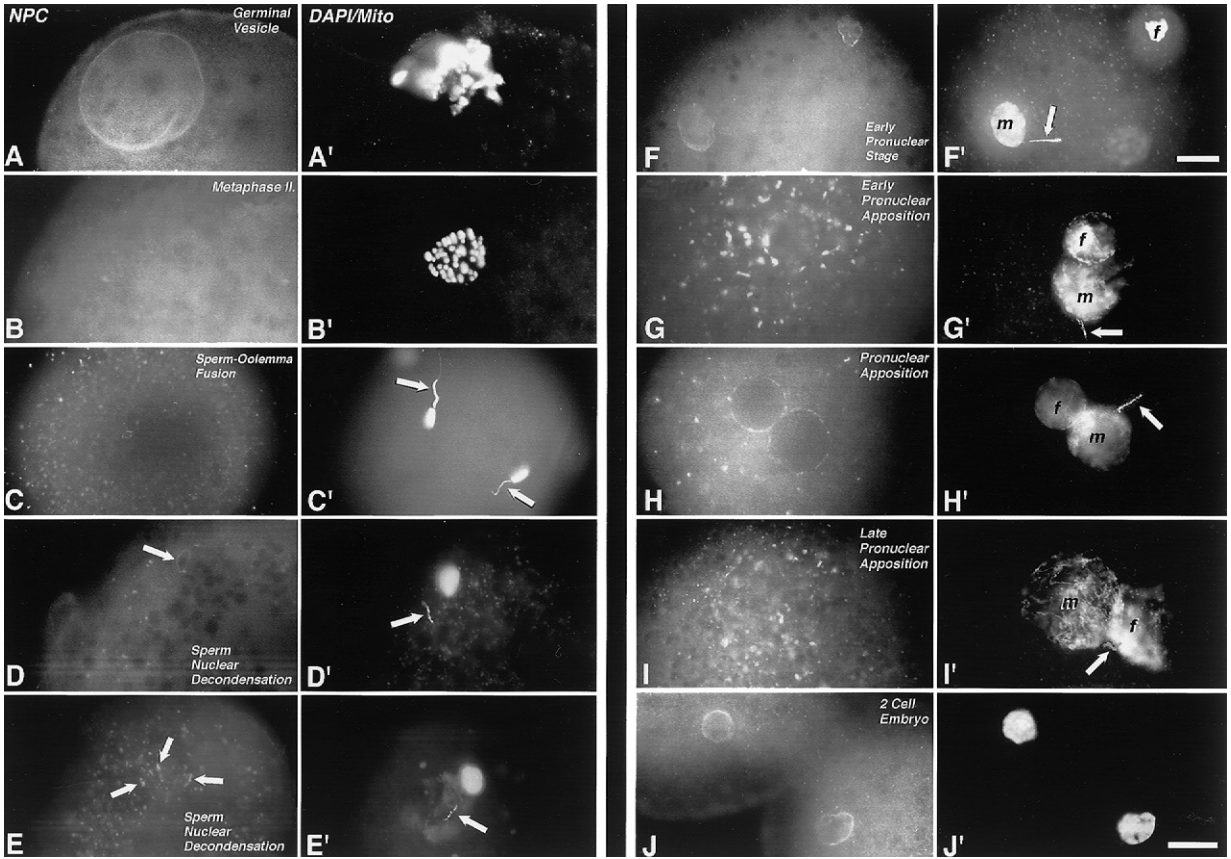
2. Formation of the Nuclear Envelope around the Developing Pronuclei

Following, or perhaps concomitantly with, the removal of the sperm plasma membrane and PT, the sperm nucleus is deprived of its nuclear envelope as it enters oocyte cytoplasm (Usui, 1996; Usui and Yanagimachi,, 1976; Usui *et al.,* 1997). Similarly, the oocyte nucleus is deprived of its NE by germinal vesicle breakdown (Stricker and Schatten, 1989). The new nuclear envelope is reconstructed around the decondensed sperm nucleus from the oocyte-derived membrane vesicles (Collas *et al.,* 1996; Pasteels, 1963; Poccia and Collas, 1996; Sutovsky and Schatten, 1997; Usui *et al.,* 1997; Vigers and Lohka, 1991). This universal step in fertilization marks the formation of nuclear and cytoplasmic compartments in the fertilized oocytes and brings about the need for establishing a bidirectional communication and exchange of molecules necessary for normal pronuclear and embryonic development. The main channel for nucleocytoplasmic transport in animal cells is the nuclear pore complex (Pante and Aebi, 1996), an assembly of the O-glycosylated proteins from the nucleoporin family. The reconstitution of the NE and the assembly of NPCs in *Xenopus* egg extracts were instrumental in dissecting the pathways leading to the assembly of functional NE (Macaulay and Forbes, 1996; Meier *et al.,* 1995; Newport, 1987; Pfaller *et al.,* 1991; Poccia and Colas, 1996). Three distinct steps with specific requirements for energy substrates are involved in the reconstitution of the nuclear envelope on the surface of ''naked'' sperm and oocyte chromatin. These include (i) an ATP- and GTP-dependent fusion of membrane vesicles into a continuous NE, (ii) a Ca^{2+}-dependent formation/insertion of NPCs into NE, and (iii) the assembly of nuclear lamina underlying the new NE (Boman *et al.,* 1992; Macaulay and Forbes, 1996; Pfaller *et al.,* 1991; Vigers and Lohka, 1992). It is a matter of ongoing discussion whether the nuclear lamina becomes assembled prior to, during, or after the assembly of the NE and NPCs. Some earlier studies showed the requirement of

nuclear lamina proteins, lamins, for the binding of membrane vesicles to chromatin (Burke and Gerace, 1986; Ulitzur *et al.,* 1992). Others, however, maintain that lamins are not necessary for NE assembly (Newport *et al.,* 1990). After fertilization, lamins A, C and lamin B become associated with both pronuclei at an early stage of pronuclear development in mice and sea urchin (Schatten *et al.,* 1985a, 1989). An accelerated expression of lamin B is seen during gastrulation in sea urchin (Holy *et al.,* 1995). Lamin B and lamin B receptor-like molecules are requrired for the formation of male pronucleus *in vitro* in sea urchins (Collas *et al.,* 1996).

Relatively little is known about the requirements and chronology of NE assembly during mammalian fertilization. Studies have demonstrated (Fig. 4) that the assembly of functional NE with NPCs occurs almost immediately after sperm incorporation and initial swelling of the sperm nucleus during bovine fertilization *in vitro* (Sutovsky *et al.,* 1998). The enlargement of both the male and the female PN and further pronuclear development depend on NPC-mediated nucleocytoplasmic transport. Ca^{2+} ions seem to be necessary for the formation of NPCs on the pronuclear envelopes of bovine zygotes, which is prevented by their depletion with the chelating agent BAPTA. In contrast, BAPTA treatment does not affect the assembly of annulate lamellae in oocyte cytoplasm, which is induced by the fertilizing spermatozoon (Sutovsky *et al.,* 1998b). Annulate lamellae (see Fig. 3J), loosely defined as the cytoplasmic stacks of NPCs (Kessel, 1992), were found in the fertilized oocytes of many mammalian species, including humans (Van Blerkom *et al.,* 1987), rhesus monkey (Sutovsky *et al.,* 1996a), cattle (Sutovsky *et al.,* 1996b), pig (Laurincik *et al.,* 1995; Szöllösi and Hunter, 1973), rabbit (Longo, 1975; Szöllösi *et al.,* 1996), and hamster (P. Sutovsky and J. Squirell, unpublished data). In bovine zygotes, the perinuclear assembly of AL appears to be controlled by the sperm aster. Consequently, the depolymerization of sperm aster microtubules blocks both the pronuclear development and the insertion of NPCs (Sutovsky *et al.,* 1998b). The dependence of pronuclear development on microtubule assembly was also demonstrated in rodents (Schatten *et al.,* 1989). The assembly of pronuclear NE in mammals appears to be regulated by the decrease of cytoplasmic MAP kinase activity (Moos *et al.,* 1995) and MPF activity (Usui *et al.,* 1997).

An alternative pathway for nucleocytoplasmic transport in mammalian zygotes was proposed by Szöllösi and Szöllösi (1988). They observed that nuclear blebbing, an evagination of the NE containing granular material, occurs frequently in mouse zygotes. Similar structures were also seen during the nuclear reprogramming of bovine zygotes reconstructed by nuclear transfer (Kanka *et al.,* 1991; Lavoir *et al.,* 1997) and in sperm-fertilized, nocodazole-treated bovine zygotes (Sutovsky *et al.,* 1998b).


NPC
Germinal Vesicle
DAPI/Mito
A
A'
Metaphase II.
B
B'
Sperm-Oolemma Fusion
C
C'
Sperm Nuclear Decondensation
D
D'
Sperm Nuclear Decondensation
E
E'
Early Pronuclear Stage
F
F'
f
m
Early Pronuclear Apposition
G
G'
f
m
Pronuclear Apposition
H
H'
f
m
Late Pronuclear Apposition
I
I'
f
2 Cell Embryo
J
J'

3. Pronuclear Development and DNA Replication

Protamine removal from the sperm nucleus occurs several hours after oocyte activation (Colleau *et al.,* 1997; Krzanovska, 1982; Kopecny and Pavlok, 1975). In addition to glutathione (Perreault *et al.,* 1984, 1988; Sutovsky and Schatten, 1997), proteins imported from oocyte cytoplasm were implicated in the sperm nuclear decondensation at fertilization in mammals and other vertebrates (Yanagimachi, 1994). Most notably, nucleoplasmin is involved in the regulation of sperm fertilization in frogs (Philpott *et al.,* 1991). Decondensing factors other than glutathione may also be present in the mammalian nucleoplasm, as sperm injected in the pronuclei of fertilized mouse oocytes decondenses in a manner similar to the decondensation of the sperm nucleus in oocyte cytoplasm (Maeda *et al.,* 1998). Chronology of pronuclear development was described *in vivo* in pig zygotes, where it starts approximately 56 h after ovulation stimulus and lasts for no more than 4 h (Laurincik *et al.,* 1995). In bovine, the pronuclear development starts 4 h after insemination and the relatively long S phase (8–10 h) starts 14–15 h after insemination (Laurincik *et al.,* 1998). The S-phase in bovine is accompanied by the appearance of nucleolus precursor bodies that, however, do not become active nucleoli until the eight-cell stage (Lavoir *et al.,* 1997; Kopecny *et al.,* 1989). The period of DNA synthesis is even longer in bovine parthenogenotes, where it lasts 12–14 h (Soloy *et al.,* 1997). Nuclear import of embryo-specific histone H-1 subtypes is required for the onset and completion of the S-phase in mammalian (Lin and Clarke, 1996) and frog (Lu *et al.,* 1997) pronuclei. Other factors from the zygotic cytoplasm may regulate DNA replication within the pronuclei. Decondensed mouse sperm nuclei introduced into two-cell blastomeres can undergo DNA repli-

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FIG. 4 Biogenesis of nuclear pore complexes (NPC) in fertilized and unfertilized bovine oocytes, as demonstrated by the triple labeling of a subset of NPC antigens with antibody mAb 414 (A–J) and by the DNA stain DAPI (A'–J') combined with the labeling of sperm tail mitochondria with a vital probe Mito Tracker Green FM (C'–J'); arrows. Oocytes were fixed at the germinal vesicle stage prior to the resumption of first meiotic division (A, A'), at the second meiotic metaphase (B, B'), at the time of sperm–oolemma binding 8 h after insemination (C, C'), shortly after sperm incorporation into oocyte cytoplasm (D, and E'), at the initial stage of pronuclear development (F, F'), at the time of pronuclear apposition (G–I'), and after the first embryonic cleavage (J, J'). Diffuse cytoplasmic labeling with mAb 414 (A, B, F, J) probably reflects the presence of a soluble cytoplasmic pool of antigens recognized by this antibody in the cytoplasm of bovine oocytes. Arrow in D points to the ring of NPC around the sperm nucleus undergoing the initial swelling in oocyte cytoplasm. Arrows in E point to the putative annulate lamellae found in the vicinity of decondensing sperm nuclei shortly after sperm incorporation into oocyte cytoplasm. m, male PN; f, female PN; arrows, sperm tails. Scale bars: 10 μm. Reprinted with permission from Sutovsky *et al.* (1998).

cation and even become transcriptionaly active (Plusa *et al.,* 1997). Such factors are also present in frog egg extracts, thus allowing the complete replication of mammalian sperm genome in a cell-free system (Xu *et al.,* 1998). To assure the synchronous pronuclear development, oocytes possess a suppressor activity that prevents female pronuclear development during the first few hours after oocyte activation (Fulka *et al.,* 1996). In turn, the development of the male PN appears to be synchronized with the completion of oocyte meiosis and female pronuclear development. Disruption of oocyte meiosis by colchicine prevented the enlargement of the male pronucleus in hamster (Wright and Longo, 1988). The persistence of the subacrosomal layer of the sperm perinuclear theca on the apical surface of the male pronucleus delayed the completion of DNA replication in the male PN and the exit from the S-phase in both male and female pronuclei after intracytoplasmic sperm injection in rhesus (Hewitson *et al.,* 1999). This effect may be due to the existence of a replication checkpoint and/or a feedback loop between the male and the female PN. Asynchronous/unequal development of the male and female PN in humans was correlated with aberrant embryonic development and an increase in mosaicism (Sadowy *et al.,* 1998). Although most stages of pronuclear development appear to be synchronized by a mechanism common for both male and female pronuclei, there is some evidence of factors involved in the development of the male, but not the female PN. This includes the male PN-specific protein kinase(s) or kinase substrates. Consequently, the development of the male, but not that of the female PN can be suppressed by the inhibitors of protein phosphorylation early after insemination, but not during the later stages of pronuclear development (Chian *et al.,* 1999). Therefore, it appears that the male and the female PN, at an early stage of fertilization, follow individual paths of development that converge into a common control mechanism before the onset of DNA replication.

IV. Other Potential Paternal Contributions

A. Elimination of Paternal Mitochondria and Sperm Tail Accessory Structures

1. Elimination of Sperm Mitochondria at Fertilization

The mitochondrial sheath of the mammalian spermatozoon is a helix of 50–75 tightly packed mitochondria that are wrapped around the sperm tail connecting piece and midpiece (Fawcett, 1975; Phillips, 1974). With the exception of the Chinese hamster (Pickworth *et al.,* 1968), the sperm tail with

an intact mitochondrial sheath enters oocyte cytoplasm during mammalian fertilization. As early as 1940, Gresson speculated about the fate of sperm mitochondria after fertilization. His conclusion that these spread across the cytoplasm and become redistributed evenly into both blastomeres at the first mitosis proved to be wrong when high-resolution biological microscopes became available. Early after sperm incorporation into the oocyte cytoplasm, an almost intact mitochondrial sheath remains attached to the sperm tail, which is maintained in close vicinity of the developing male pronucleus (Sutovsky *et al.,* 1996b). As the pronuclear development advances, the sperm mitochondria become swollen and eventually detach from the disintegrating axoneme. Such mitochondria are surrounded by multivesiculated bodies and lysosomes and their crystae degenerate and later completely disappear (Hiraoka and Hirao, 1988; Sutovsky *et al.,* 1996a,b; Szöllösi, 1965). Using the vital mitochondrion-specific dye MitoTracker, we traced bull sperm mitochondria in the cytoplasm of one-, two-, and four-cell bovine embryos and recorded the degenerative changes of the mitochondrial sheath occurring before its disappearance at the four-cell stage (Sutovsky *et al.,* 1996b; Fig. 5). These results were followed up and confirmed by studies in mice, where the MitoTracker-labeled sperm tails injected into the cytoplasm of mature oocytes also disappeared at the four-cell stage (Cummins *et al.,* 1997). Using a specific antibody against a sperm mitochondrial membrane component, Shalgi *et al.* (1994) observed the loss of sperm mitochondrion labeling at the two-cell stage in mouse and at the four-cell stage in the rat. Kaneda *et al.* (1995) reported the loss of membrane potential in sperm mitochondria inside pronuclear stage mouse eggs. It appears that sperm mitochondria become obsolete after sperm incorporation into oocyte cytoplasm, as their destruction by cyanide poisoning prior to *in vitro* fertilization by intracytoplasmic sperm injection (ICSI) has no effect on further embryonic development (Ahmadi and Ng, 1997). We can therefore conclude that sperm mitochondria are eliminated by the action of oocyte cytoplasm at an early stage of mammalian embryogenesis.

2. Strictly Maternal Inheritance of mtDNA in Mammals: Why Is It Necessary?

Each mitochondrion, including those of the mammalian sperm tail, carries up to 10 complete copies of mitochondrial genome encoding the main components of the mitochondrial respiratory chain (Cummins, 1998; Zeviani and Antozzi, 1997). In contrast, proteins of the mitochondrial membrane, as well as some other mitochondrial respiratory enzymes, are encoded by the nuclear genes, from which the mitochondrial genes differ substantially. Briefly, mitochondrial genes (i) possess a very simple and inefficient repair mechanism, making them prone to mutagenesis; (ii) are

not replicated under the control of the cell cycle, which explains the high rate and relatively fast accumulation of mutated mtDNA; (iii) exist in multiple copies within each cell; (iv) lack the protective histones and DNA-binding core proteins; (v) are inherited uniparentaly (maternally) in mammals; and (vi) do not undergo homologous recombination. These properties (Zeviani and Antozzi, 1997) account for the high rate of mutagenesis seen in mitochondrial DNA and suggest that propagating only the maternal mtDNA line might be an evolutionary advantage (Boore, 1997; Hurst, 1992; Hurst *et al.,* 1996; Jenuth *et al.,* 1997; Marchington *et al.,* 1997). In fact, the uniparental inheritance of mtDNA occurs in most live eukaryotes, including plants and protozoans (Birky, 1995). The most likely reason for the elimination of sperm mitochondria in mammals is the high probability that their mtDNA will be damaged during insemination and fertilization (Max, 1992). Reactive oxygen species produced by epithelial cells, leukocytes, and aberrant spermatozoa (Aitken *et al.,* 1994, 1998) have the ability to induce mtDNA mutations (Yakes and Van Houten, 1997), even though the mitochondrial sheath of the mammalian spermatozoon is protected by an extensive disulfide bond cross-linking that takes place during the epididymal passage (Bedford, 1979; Calvin and Bedford, 1971). Such damage is not likely to be repairable by the simple DNA repair mechanism of mitochondria (Zeviani and Antozzi, 1997). In addition to epigenetic factors, mtDNA mutations and mitochondrial diseases appear to be more prevalent and more severe in males than in females (Frank and Hurst, 1996). Using a highly sensitive ''long'' polymerase chain reaction technique, a study in humans demonstrated an unexpectedly high rate of sperm mtDNA mutations, as compared to a low rate of mutated mtDNA in oocytes (Reynier *et al.,* 1998). Thus, the destruction of sperm mitochondria after fertilization may prevent the propagation of mutated paternal mtDNA and mitochondrial diseases (Ankel-Simon and Cummins, 1996; Cummins, 1998). The replication and accumulation of such mutated paternal mtDNA in the embryonic cytoplasm could lead to the coexistence of two different mitochondrial genomes in the cytoplasm of a single cell, the condition known as heteroplasmy. These two mitochondrial genomes may be separated by mitotic segregation into two distinct cell lineages, each containing only one type of mtDNA (Zeviani and Antozzi, 1997). Although a certain degree of heteroplasmy caused by the aging-related accumulation of the point mutations and rearrangements in mtDNA appears to be normal (Cortopassi *et al.,* 1992; Hattori *et al.,* 1991), heteroplasmy has been implicated in a wide array of disorders. These most frequently affect tissues that rely heavily on mitochondrial respiration, such as the visual and auditory system, nervous system, muscles, heart, pancreas, kidney, and liver (Lightlowers *et al.,* 1997; Sherratt *et al.,* 1997; Zeviani and Antozzi, 1997; Wallace, 1994).

Reproductive disorders that affect spermiogenesis, sperm motility, and formation of the sperm mitochondrial sheath were linked to mtDNA deletions and mutations (Cummins *et al.,* 1994; Kao *et al.,* 1995, 1998; Lestienne *et al.,* 1997). Relatively little is known about the heteroplasmy produced by paternally inherited mtDNA, as studies of interspecies mouse crosses (Gyllensten *et al.,* 1991; Kaneda *et al.,* 1995), the only ones to demonstrate the paternal transfer of mtDNA in mammals after natural fertilization, did not evaluate the fitness of such offspring.

3. Putative Mechanism of Sperm Mitochondrion Recognition and Elimination

Several theories were formulated to explain the species-specific destruction of sperm mitochondria inside the mammalian embryonic cytoplasm. The theory of mtDNA dilution (Ankel Simon and Cummins, 1996; Smith and Alcivar, 1993) calculated the high theoretical ratio of paternal versus maternal mitochondria at fertilization (1:1000), but failed to explain the transmission of paternal mtDNA occurring at a relatively high rate in the interspecies crosses (Gyllensten *et al.,* 1991; Kaneda *et al.,* 1995). Based on the assumption that sperm mitochondria suffer oxidative damage during their passage through female reproductive tract and oocyte vestments, Allen (1996) suggested that such damaged mitochondria or mitochondrial memberanes are recognized in the oocyte cytoplasm and destroyed. Although this theory provides a feasible explanation for why the sperm, but not the oocyte mitochondria, are destroyed after fertilization, it once again does not explain the failure of oocyte cytoplasm to recognize and destroy sperm mitochondria in the interspecies crosses of *Mus musculus* and *Mus spretus* (Gyllensten *et al.,* 1991; Kaneda *et al.,* 1995). The study by Kaneda *et al.* (1995) also demonstrates that mitochondrial proteins, but not the mtDNA itself, are recognized by the mechanism responsible for the elimination of sperm mitochondria. Sperm mitochondria of a congenic strain of mice featuring mitochondrial membrane proteins of *M. musculus* and mtDNA from *M. spretus* are destroyed efficiently after fertilization and no transfer of paternal mtDNA occurs therein (Kaneda *et al.,* 1995). Therefore, it is likely that one or more male germ cell-specific antigens or signaling peptides, which are not present in the oocyte mitochondria, serve for the recognition of sperm mitochondria by the oocyte cytoplasm.

Our observations of sperm mitochondria being destroyed inside the bovine embryonic cytoplasm (Sutovsky *et al.,* 1996b; Fig. 5) suggested that the elimination of paternal mitochondria depends on the progression of the embryonic cell cycle. This led to the suggestion that ubiquitin, a small 8.5-kDa proteolytic polypeptide (Goldstein *et al.,* 1975; Ciechanover *et al.,* 1984) that controls the cell cycle through the destruction of the cyclin

component of MPF at the metaphase/anaphase transit (Glotzer *et al.,* 1991), could be involved in the elimination of paternal mitochondria at fertilization. New findings (Sutovsky *et al.,* 1998a; Fig. 6) demonstrate the binding of ubiquitin to the sperm mitochondria incorporated in the cytoplasm of bovine oocytes. Ubiquitin and ubiquitin-conjugating enzyme E2 were also found in round and elongated spermatids and testicular spermatozoa. Disulfide bond-reducing treatment was necessary to reveal ubiquitin in the mitochondria of ejaculated bull spermatozoa (P. Sutovsky *et al.,* unpublished data).

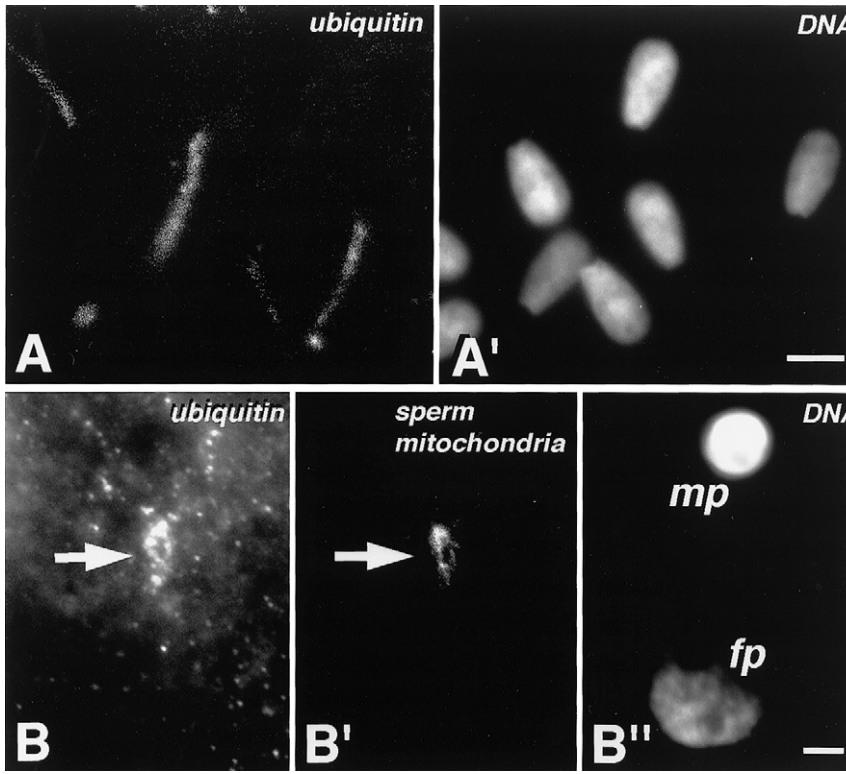


FIG. 6 Ubiquitination of bull sperm mitochondria before (A, A') and after *in vitro* fertilization. Ubiquitin tagging of bull sperm mitochondria (A) was revealed by the amplification of anti-ubiquitin antibody labeling with the biotin–avidin procedure. (A') DNA staining of sperm nuclei with DAPI. Polyubiquitination (arrow) of the sperm mitochondria after *in vitro* fertilization was demonstrated in a pronuclear egg with a simple two-step labeling procedure with an anti-ubiquitin antibody (B) followed by a rhodamine-conjugated secondary antibody. Sperm mitochondria (B'; arrow) were prelabeled with a green fluorescent, vital probe MitoTracker prior to *in vitro* fertilization. DNA in the male (mp) and female (fp) pronuclei was stained with DAPI Scale bars: 5 μm. P. Sutovsky, unpublished results.

Most cytosolic and even some membrane proteins are prone to ubiquitination (Ciechanover, 1994; Bonifacino and Weissman, 1998), which starts by the activation of the 8.5-kDa ubiquitin molecule with activating enzyme E1, followed by the conjugation of the activated ubiquitin to the terminal NH_2 group of a lysine residue of the substrate, that is assisted by the ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 (Ciechanover, 1994; Jentsch, 1992). Subsequently, multiple ubiquitin molecules are added to the monoubiquitinated substrate, forming a polymer-like ubiquitin tag that guides the target protein to the site of destruction in either a proteasome or a lysosome (Chau *et al.,* 1989). Ubiquitination can occur either in a nonspecific manner where the lysine residua of misfolded or damaged proteins are ubiquitinated or in a ligand-specific manner (Bonifacino and Weissman, 1998). According to our scenario of mitochondrial ubiquitination, sperm mitochondria are tagged by few ubiquitin molecules during spermiogenesis, most likely at the secondary spermatocyte or round spermatid stage (Fig. 6A). This is consistent with the finding of increased ubiquitination activity in rooster, mouse, and human round spermatids (Agell and Mezquita, 1988; Tipler *et al.* 1997) and with the presence of the ubiquitin-conjugating enzyme E2 in mouse, rat, and human round spermatids (Tipler *et al.,* 1997; Wing and Jain, 1995; Wing *et al.,* 1996). Disulfide bond cross-linking may temporarily mask the ubiquitin tag during epididymal passage and uterotubal transport. Following the incorporation of the sperm mitochondrial sheath into oocyte cytoplasm and the relief of disulfide bonds masking the ubiquitin tag of sperm mitochondria, the oocyte-derived ubiquitin molecules may recognize the ubiquitin tag on the mitochondrial membranes (Fig. 6B), and the long polyubiquitin chains may be attached to sperm mitochondria in order to provide the recognition site for lysosomes and/or proteasomes.

Mitochondrial proteins rich in lysine, an attachment site amino acid of ubiquitin (Ciechanover, 1994; Jentsch, 1992; Jentsch and Schlenker, 1995), are the most likely substrates for ubiquitination. Such proteins were isolated from bull (Pallini *et al.,* 1979) and mouse (Kleene *et al.,* 1990; Cataldo *et al.,* 1996) spermatozoa and appear to be localized in the outer mitochondrial membrane, which makes them suitable candidates for ubiquitin substrates. Both bull and mouse mitochondrial proteins are extremely rich in cysteine, accounting for 21.2% of all amino acids in mouse SMCP-protein and 17.9% in its bull analog (Kleene *et al.,* 1990; Pallini *et al.,* 1979), and lysine, representing 11.2 and 11.3% of all amino acids in mouse and bull SMCP, respectively. The high content of cysteine residues responsible for the formation of disulfide bonds may explain why disulfide bond reduction is required to obtain the binding of ubiquitin-specific antibodies to the mitochondrial sheath of ejaculated (see Fig. 6A), but not testicular, spermatozoa. Disulfide bond cross-linking occurring during epididymal maturation lends rigidity to the mitochondrial sheath of the mammalian spermatozoa (Bed-

ford, 1979). Distinct surface domains seen on the adjacent mitochondrial surfaces, in the submitochondrial reticulum, and on the mitochondrial surfaces facing the plasma membrane can be perturbed by DTT treatment of the sperm (Olson and Winfrey, 1992).

4. Dangers of Foreign mtDNA Transmission for Reproductive Biotechnologies

Characterization of the pathways leading to the destruction of paternal mitochondria at fertilization has important implications for the new methods of assisted procreation, including nuclear transfer and cloning, oocyte cytoplasm donations, intracytoplasmic sperm injection, and round spermatid injection (reiewed in more detail in Section VII). Using each of these methods, foreign mitochondria are introduced into oocyte cytoplasm where they may reproduce autonomously unless they are recognized and processed properly by the cytoplasmic machinery of the oocyte. It is not known whether these foreign, i.e., somatic or germ cell, mitochondria can be eliminated by the oocyte in a fashion identical to the elimination of mature sperm mitochondria during natural fertilization. The persistence of two different mitochondrial genomes in the cytoplasm of a single cell, a condition referred to as heteroplasmy, may collide with the development of such embryos and result in severe anomalies. Abnormal mitochondria with ruffled membranes and multiple granular inclusions in the mitochondrial matrix were observed after nuclear transfer in bovine (King *et al.,* 1996). These authors suggest that mitochondria carried over from a transferred cell could impair the development of those embryos. Heteroplasmy was found in cattle (Meirelles *et al.,* 1999; Plante *et al.,* 1992) and mice (Smith *et al.,* 1991) embryos produced by nuclear transfer, suggesting that the mitochondrial genome from the donor cells was incorporated efficiently into the reconstructed embryos and replicated. A contradictory report, however, found no foreign mitochondria or mtDNA in mice derived from zygotes microinjected with isolated mitochondria from testis or liver (Ebert *et al.,* 1989). It is therefore not clear whether the oocyte cytoplasm can reliably detect and eliminate foreign mitochondria originating in cells other than mature spermatozoa. Phenotypic defects were reported in hybrid mouse embryos created by nuclear transfer (Reik *et al.,* 1993). Eight cloned claves were born in Japan and four of them died shortly after birth (Kato *et al.,* 1998). Because there were no obvious malformations involved, the authors suggested that epigenetic factors, including effects ''possibly stemming from mitochondrial or maternal gene products,'' contribute to the poor fitness of the cloned offspring. Perhaps the cells used for cloning and nuclear transfer should be completely deprived of cytoplasm and mitochondria prior to the micromanipulation or at least treated with agents capable

of damaging the mitochondria, such as sodium cyanide (Ahmadi and Ng, 1997). Such knowledge is crucial for the development of safe and effective techniques of nuclear transfer and cloning, where the cytoplasmic inheritance derived from mtDNA is correlated to the fitness and performance of farm animals (Smith and Alcivar, 1993).

Progress in the *in vitro* culture, transplantation (Brinster and Avarbock 1994; Clouthier *et al.,* 1996), and intracytoplasmic injection/fusion (Kimura and Yanagimachi, 1995a,b; Ogura *et al.,* 1997; Sasagawa *et al.,* 1998) of spermatogonia and spermatocytes holds promise for their ability to utilize diploid spermatogenic cells for the amelioration of animal production and for the treatment of human infertility. However, the effect of cytoplasmic inheritance on such reconstructed embryos has yet to be determined. It should also be remembered that the aforementioned micromanipulations include chemical treatments and mechanical perturbations that could easily affect the mutation-prone mtDNA. Transfer of annucleate donor cytoplasm into recipient oocytes and subsequent *in vitro* fertilization resulting in a birth of a baby (Cohen *et al.,* 1997) raised concerns about the possibility of creating heteroplasmy by human oocyte cytoplasm donation (St. John and Barrat, 1997). Similarly, ICSI in humans and animals may pose an increased risk of the transmission of defective paternal mtDNA to the progeny (St. John *et al.,* 1997). Only detailed knowledge of the pathway(s) leading to the elimination of foreign mitochondria by oocyte cytoplasm can lead to the creation of genetically safe technologies for intra- and interspecific nuclear transfer and intracytoplasmic injection of spermatozoa and spermatogenic cells.

5. Destruction of Outer Dense Fibers and Microtubule Doublets in Embryonic Cytoplasm

The perm axoneme is a complex structure composed of 9 + 2 arrangement of microtubule doublets, paralleled by nine outer dense fibers (ODF) in the axonemal principal piece and midpiece. In the connecting piece, serving for the attachment of the axoneme to the basal plate of the sperm nucleus, the ODF are transformed into nine striated columns caging the capitulum-embedded sperm centriole. The principal piece is covered with the fibrous sheath (FS) and the midpiece/connecting piece complex is enveloped by the mitochondrial sheath (Fawcett, 1975; Sutovsky *et al.,* 1996b; Sutovsky and Schatten, 1997). During fertilization, the uppermost mitochondria are removed from the mitochondrial sheath, thus unmasking the connecting piece columns that are subsequently excised from the sperm nucleus and eventually dismantled (Sutovsky *et al.,* 1996a,b). This event leads to the release of the sperm centriole in the zygotic cytoplasm and to its transformation into an active zygotic centrosome (Le Guen and Crozet, 1989; Sutovsky

et al., 1996b). Similarly, the FS dissolves within a few hours after sperm incorporation in bovine (Sutovsky *et al.,* 1996b). ODF and the mitochondrial sheath appear to persist in embryonic cytoplasm well beyond the first embryonic cleavage (Hiraoka and Hirao, 1988; Sutovsky *et al.,* 1996b). Microtubule doublets can persist in oocyte cytoplasm for several embryonic cell cycles (Austin, 1961; Simerly *et al.,* 1993a: Szöllösi, 1976). The disassembly of the mitochondrial sheath and striated columns may be facilitated by oocyte glutathione (Sutovsky and Schatten, 1997), and the dismantling of the connecting piece may be mediated by phosphorylation/dephosphorylation events (Pinto-Correia *et al.,* 1994; Long *et al.,* 1997) and by the calcium-binding protein centrin (Fechter *et al.,* 1996). Mechanisms participating in the destruction of FS and ODF are not known, although it appears that these structures are not ubiquitinated as are sperm mitochondria (P. Sutovsky *et al.,* unpublished data). It is possible that kinases and other enzymes bound to the axonemal structures contribute to this process. The fibrous sheath contains the enzyme hexokinase (Kalab *et al.,* 1994) and the A-kinase-anchoring protein (AKAP; Carrera *et al.,* 1994). Characterization of the individual steps leading to the disassembly of the sperm tail in oocyte cytoplasm may explain why fertilization arrests (Asch *et al.,* 1995; Simerly *et al.,* 1995; Hewitson *et al.,* 1996) occur after sperm entry into oocyte cytoplasm.

B. Significance of Paternal Contributions for Assisted Fertilization

1. Intracytoplasmic Sperm Injection

By microinjecting an intact spermatozoon directly into oocyte cytoplasm, intracytoplasmic sperm injection bypasses multiple steps of natural fertilization, including zona penetration, sperm oolemma fusion, and sperm incorporation. Because these steps are viewed as Nature's way to weed out aberrant spermatozoa and select the most viable ones for fertilization, such treatment imposes substantial risks on the development of resultant zygotes (Schatten *et al.,* 1998). Aside from having the potential to propagate aberrant male genomes, ICSI could produce embryonic abnormalities as a result of the persistence of the intact sperm accessory structures that would normally be eliminated before the sperm entry into oocyte cytoplasm during natural fertilization. On the other side, one has to weigh the obvious benefits, including a dramatic rise in the successful treatment of idiopathic infertility in humans, the potential of ICSI for the creation of farm animals with a modified genome, and for the preservation of endangered animal species and rare breeds. Mice (Kimura and Yanagimachi, 1995c), rabbit

(Iritani and Hoshi, 1989), and primates, including humans (Palermo *et al.,* 1992; Van Steiterghem *et al.,* 1993) and monkeys (Hewitson *et al.,* 1996, 1998; Ogonuki *et al.,* 1998; Sutovsky *et al.,* 1996a; Fig. 7), show consistently high rates of fertilization, development, and birth after ICSI. In contrast, the method is substantially less efficient in rat (Dozortsev *et al.,* 1998) and in farm animals such as cattle (Goto *et al.,* 1990; Rho *et al.,* 1998), horse (Dell'Aquila *et al.,* 1997; Grondahl *et al.,* 1997), sheep (Catt *et al.,* 1996), pig (Catt and Rhodes, 1995), and goat (Keskintepe *et al.,* 1997). Success rates of ICSI in the individual mammalian species are probably correlated with the morphology of their sperm and oocytes. For example, ICSI is very difficult in bovine, a species with a thick, extraction-resistant perinuclear theca (Perreault *et al.,* 1988; Sutovsky *et al.,* 1997a,c) and a viscous cytoplasm rich in lipid inclusions. Based on previous studies on the role of disulfide bond reduction in male pronuclear development (Perreault *et al.,* 1984; Sutovsky and Schatten, 1997), Rho *et al.* (1998) used the combination of disulfide bond-reducing pretreatment of the sperm and drug-induced oocyte activation to increase the success rate of bovine ICSI. Our earlier studies in bovine showed the ability of the reducing agent DTT to recover male pronuclear development in oocytes that were experimentally depleted of their intrinsic reducing agent glutathione (Sutovsky and Schatten, 1997). Presuming that some of the oocyte activation factors, oscillogens, are anchored to sperm perinuclear theca (see Section III,C), one could explain the necessity for the parthenogenetic activation of bovine oocytes after ICSI by the limited/retarded dissolution of intact bull sperm PT in the highly viscous cytoplasm of bovine oocytes. Removal of the plasma membrane and structural/biochemical changes of PT during sperm incorporation that do not occur during ICSI (Sutovsky *et al.,* 1997a) may facilitate its dissolution in oocyte cytoplasm during natural fertilization. Identification and molecular cloning of PT-bound oscillogens followed by the production of their recombinant analogs could be useful for increasing the success rate of ICSI in farm animals and for treating the infertility in globozoospermic men, whose sperm lack both PT (Escalier, 1990) and the potential to induce oocyte activation after ICSI (Battaglia *et al.,* 1997; Rybouchkin *et al.,* 1996). Even though the primate PT is more susceptible to disulfide bond reduction than that of *Arterodactilia* (Sutovsky *et al.,* 1997c), the persistence of subacrosomal PT after ICSI has been observed in rhesus monkeys (Hewitson *et al.,* 1996, 1999; Sutovsky *et al.,* 1996a; Fig. 7 and Fig. 9A) and humans (Bourgain *et al.,* 1998; Küpker *et al.,* 1998; Sathananthan *et al.,* 1997a), where it was interpreted as the persistence of the whole acrosome. Although the acrosomal content appears to be harmless after ICSI in humans (Satahananthan *et al.,* 1997a), the persistence of subacrosomal PT could cause retarded or blocked sperm nuclear decondensation in both humans and rhesus. Our study in rhesus demonstrated that the residual PT, which forms the cap on

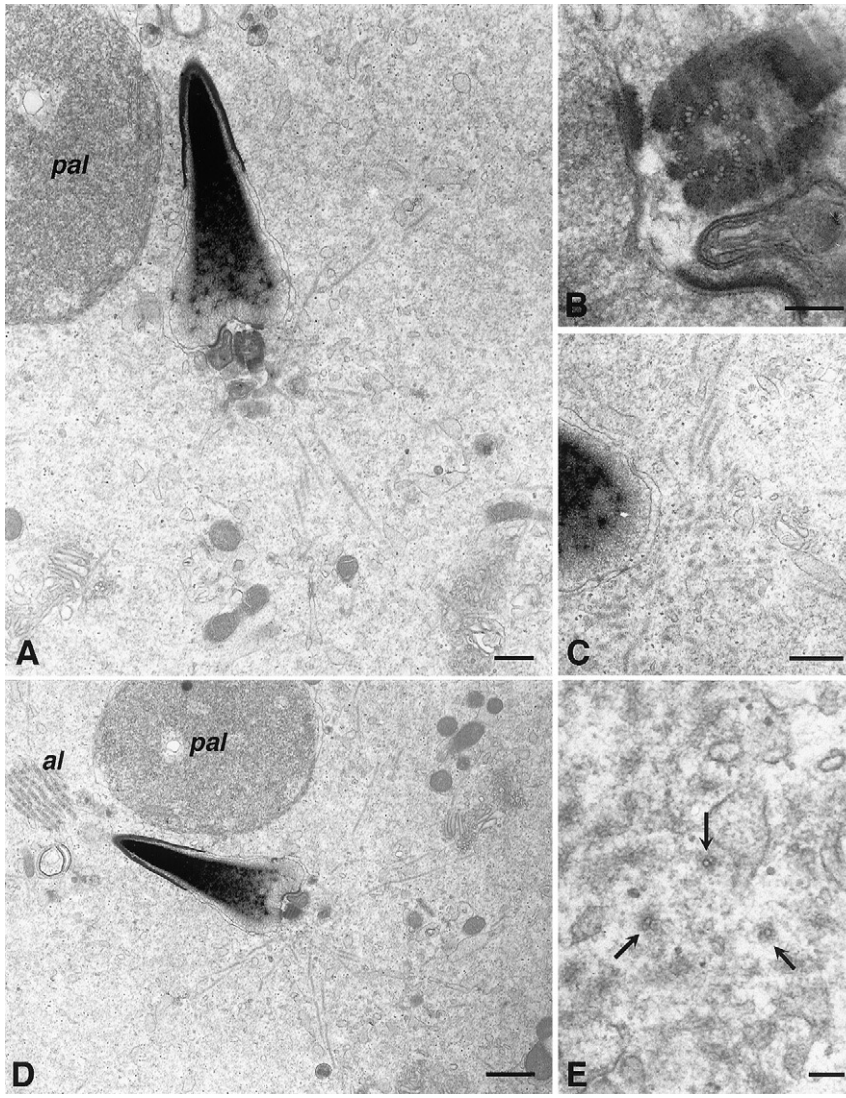


FIG. 7 Sperm aster formation in an ICSI-fertilized egg, illustrated by three serial sections (A + B, C + E, and D) of the same injected sperm. Single sperm aster microtubules (A, D) grow from the proximal centriole (B; detail of A) of the injected sperm. Occasionally, microtubule doublets (C, E; arrows) occur in the sections immediately following the cross section of centriolar triplets. The minus ends of individual microtubules are also seen in the electron-dense mass over the pericentriolar region of this sperm. Note the presence of annulate lamellae (al) and their precursor body (pal) near the nucleus of the injected sperm in A and D and the persistence of the subacrosomal perinuclear theca on the apex of the sperm nucleus. Scale bars: 1 μm in D, 500 nm in A and C, 200 nm in B, and 100 nm in E. Reprinted with permission from Sutovsky *et al.* (1996).

the apical surface of the developing male pronucleus, can prevent DNA replication in the male and indirectly, probably through a replication-related checkpoint, in the female pronucleus (Hewitson *et al.,* 1999). Such uneven decondensation of the sperm nucleus/male PN also raises concerns about chromosome partition at first mitosis. Although ICSI in humans produces the overall rates of chromosomal abnormalities similar to those of the general population, the rates of sex chromosome abnormalities after ICSI are doubled (Bonduelle *et al.,* 1998; Flaherty *et al.,* 1995; In't Veld *et al.,* 1995; Macas *et al.,* 1996a,b; Persson *et al.,* 1996; Simpson, 1998). We suspect that the residual PT (Figs. 7A, 7D, and 9A) may be a part of this problem, as the Y chromosome in most human spermatozoa is located in the apical region of the nucleus that is sheltered by subacrosomal PT known to persist after ICSI (Luetjens *et al.,* 1999). Tateno and Kamiguchi (1999) implicated the untimely sperm head decondensation in the occurrence of structural chromosomal abnormalities during cross-fertilization between Chinese and Syrian hamsters. Despite the initial concerns, the inheritance of the centrosome after ICSI appears to follow the path of natural fertilization in all mammals studied to date (Simerly *et al.,* 1996; Navara *et al.,* 1996b). Mechanical disruption of the sperm tail plasma membrane and of the accessory structures covering the connecting piece and centriole may be helpful in achieving a high success rate after ICSI in humans and other species with paternally inherited centrioles (Catt and O'Neill, 1995; Van den Bergh *et al.,* 1995). Concerns were raised regarding the possibility of paternal mtDNA transfer by ICSI (St.John *et al.,* 1997), but preliminary evidence suggests that this does not occur in humans (Torroni *et al.,* 1998).

2. Round Spermatid Injection and Transfer of Diploid Spermatogenic Cells into Oocyte Cytoplasm

Round spermatid injection (ROSI) uses an immature haploid spermatogenic cell, round spermatid, instead of a mature testicular or ejaculated spermatozoon for microinjection into oocyte cytoplasm. The technique has been used successfully in mice (Kimura and Yanagimachi, 1995a; Sato *et al.,* 1998) where live and viable offspring were obtained. Birth of live pups after ROSI was also reported in rabbit (Sofikitis *et al.,* 1996), and formation of a sperm aster-like microtubule arrangement and embryonic cleavage was reportedly obtained in pig (Lee *et al.,* 1998). Human babies born after ROSI have been reported (Barak *et al.,* 1998; Fishel *et al.,* 1995; Tesarik *et al.,* 1995), although reexamination (Fishel *et al.,* 1996; Tesarik, 1997; Silber *et al.,* 1998) suggests that elongated spermatids, and even abnormal ejaculated spermatozoa, could have been mistaken for round spermatids in such reports. This controversy is due in part to a vague definition of a round spermatid in clinical studies that encompasses a variety of stages,

including those that fit in the elongated spermatid category. Clinical reports often state that an elongated spermatid was used to obtain pregnancies (Antinori *et al.,* 1997; Bernabeau *et al.,* 1998; Sofikitis *et al.,* 1998; Vanderwalzeman *et al.,* 1997). Therefore, it appears that ROSI performs well in animal, rodent models with a strictly maternal mode of centrosomal inheritance, but the elongating or elongated spermatid stage has to be used in order to obtain pregnancies in infertility patients.

What is the difference between a round and an elongating/elongated spermatid that makes the latter, but not the former, capable of participating in the development? At the round spermatid stage (Fig. 8), the sperm accessory structures start to form, including the acrosome, perinuclear theca, and the sperm axoneme (Oko and Clermont, 1998). Two centrioles are seen in round spermatids of rodents, ruminants, and primates (Fouquet *et al.,* 1998; Manandhar *et al.,* 1998; P. Sutovsky, unpublished data; Fig. 8). The proximal centriole occupies the implantation fossa of the condensing spermatid nucleus and probably participates in the formation of a sperm tail connecting piece and in the attachment of the sperm tail to the nucleus. The distal centriole seems to serve as a mold for the formation of axonemal microtubule doublset, not unlike the cilia seen in *Protozoa* (Sanders and Salisbury, 1989). It is not until spermatid elongation that the reduction of the distal centriole takes place (Fouquet *et al.,* 1998; Manandhar *et al.,* 1998). The proximal centriole is retained in most nonrodent mammals (see Table II for references). Thus the developmental roles of either of the two round spermatid centrioles may not be compatible with that of an organizer of the zygotic centrosome. In contrast, the proximal centriole of an elongated spermatid with a well-defined axoneme and connecting piece may already be capable of converting itself into an active, microtubule-nucleating zygotic centrosome, if introduced into oocyte cytoplasm. Similarly, the ability to activate the oocyte seems to be acquired at the beginning of spermatid elongation (Sofikitis *et al.,* 1997), perhaps as a result of the completion of perinuclear theca morphogenesis and/or posttranslational processing of the oscillogenic proteins in it. Mitochondrial sheath differentiation starts at the round spermatid stage and follows the differentiation of the axoneme during spermatid elongation. It follows that the round spermatid should be defined as a haploid, round-shaped spermatogenic cells featuring (i) an acrosomal granule or acrosomal cap/vesicle that may be connected to a still decondensed sperm nucleus, (ii) mitochondria that can be loosely associated or polarized, but not yet connected to each other in a nascent mitochondrial sheath, (iii) two intact centrioles, composed of nine microtubule triplets, that are not connected to each other by the striated extensions of the axonemal outer dense fibers, (iv) a perinuclear theca that covers the apical surface of the nucleus at the developing subacrosomal region or perforatorium, but not the posterior region of the nucleus (future postacro-

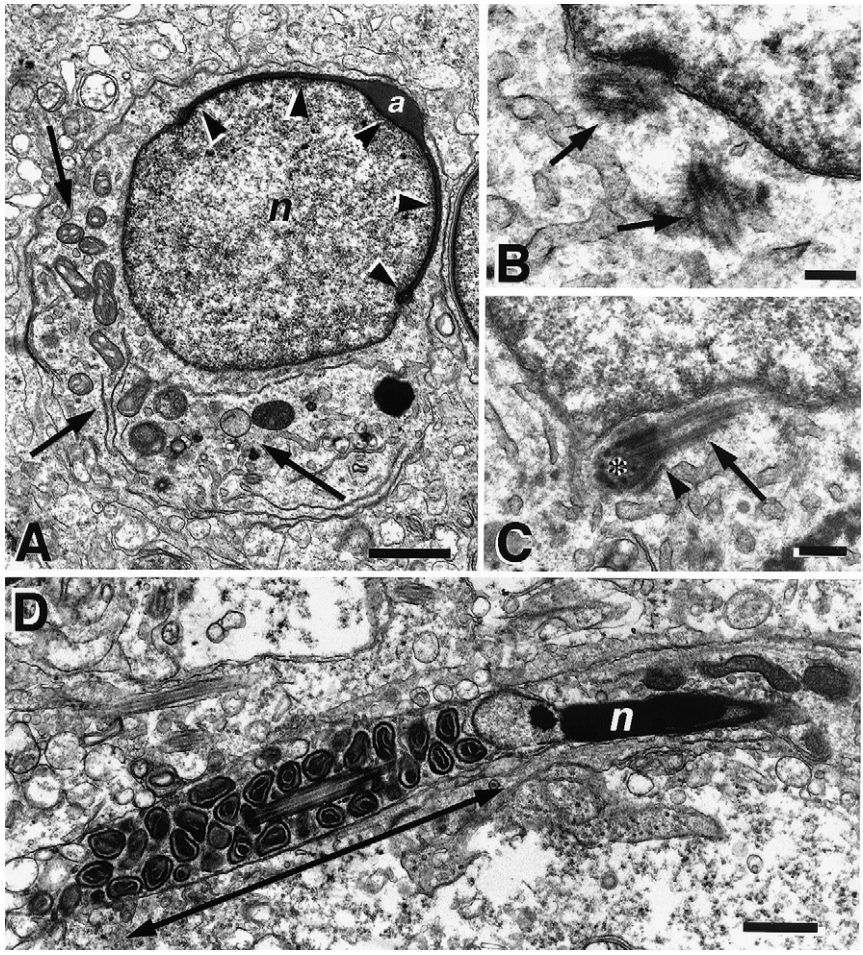


FIG. 8 Ultrastructural characteristics of round and elongated spermatids in rhesus monkey. (A) A round spermatid featuring the nucleus (n) with an acrosomal cap (a) and an underlying subacrosomal layer of perinuclear theca (arrowheads). Note the polarization of cytoplasm and mitochondria (arrows). (B) Two centrioles (arrows) of a round spermatid. The proximal centriole (upper left side) is already attached to the implantation fossa of the nucleus, whereas the distal centriole (lower right) is still in the cytoplasm. (C) Formation of the sperm axoneme at the beginning of spermatid elongation; the proximal centriole (asterisk) is surrounded by the nascent capitulum, and the distal centriole (arrowhead) is engaged in the enucleation of axonemal microtubules (arrow). (D) An elongated spermatid with an almost fully condensed nucleus (n) and an axoneme enveloped by the developing mitochondrial sheath (arrow). Scale bars: 1 μm in A, 200 nm in B and C, and 2 μm in D. (P. Sutovsky, unpublished data.)

somal sheath), and (v) an amount of cytoplasm that equals approximately one-half of that of a secondary spermatocyte. In addition to the developmental incompetence of round spermatid centrioles and the absence or nascent inactive state of the oscillogens, the persistence of the nascent PT on the injected spermatid nucleus may contribute to the failures of ROSI in a way similar to the persistence of PT after ICSI (Fig. 9B; P. Sutovsky and T. Dominko, unpublished data). The presence of an intact plasma membrane and cytoplasm, unless removed by permeabilization (hence the ROSI modification called round spermatid nucleus injection or ROSNI), may also delay the ''processing'' of the injected spermatid by oocyte cytoplasm and reduce the success rate of ROSI. In contrast, sperm mitochondria appear to be recognized by oocyte cytoplasm and destroyed in a manner similar to natural fertilization after ROSI in mice (Cummins *et al.,* 1998) and bovine (Fig. 9C; P. Sutovsky and T. Dominko, unpublished results).

Timing may be of importance for the success of ROSI and spermatocyte injection. Ogura *et al.* (1997) demonstrated that the primary spermatocytes

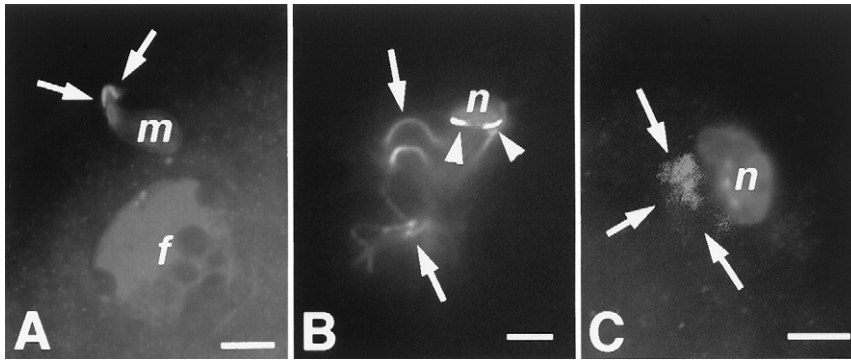


FIG. 9 Persistence of sperm and spermatid accessory structures after intracytoplasmic sperm injection (ICSI) in rhesus monkey (A) and round spermatid injection (ROSI) in bovine (B, C) as shown by indirect immunofluorescence. (A) The persistence of the subacrosomal perinuclear theca (arrows) on the apical surface of an aberrant male pronucleus (m) that would otherwise reach the diameter of the female pronucleus (f). (B) The nucleus (n) of the bull round spermatid bears the remnants of the nascent perinuclear theca (arrowheads) 20 h after injection into the bovine oocyte cytoplasm. Note the fragments of the sperm tail (arrows) already present in the cytoplasm of this late round/early elongated spermatid. (C) Presence of clustered paternal mitochondria (arrows) in the cytoplasm of a bovine oocyte 20 h after fusion with a round spermatid. n, round spermatid nucleus. Sperm accessory structures were labeled after fixation in formaldehyde and permeabilization in Triton X-100 with specific antibodies against the perinuclear theca (A, B) and microtubules (B) followed by appropriate fluorescently tagged secondry antibodies. DNA (A–C) was stained with blue fluorescent stain DAPI, and round spermatid mitochondria (C) were prelabeled with a vital fluorescent probe MitoTracker CMTM Ros prior to microinjection. Scale bars: 5 μm. P. Sutovsky and T. Dominko, previously unpublished data.

of mouse injected into prophase I oocytes undergo nuclear envelope breakdown and chromosome condensation as a consequence of high MPF activity in the cytoplasm of maturing oocytes. In contrast, Usui *et al.* (1999) have shown that the nucleus of a spermatocyte or spermatid, electrofused with a metaphase II-arrested hamster oocyte, does not undergo substantial morphological changes, probably because such oocyte cytoplasm loses its MPF activity before it can reach the nucleus of the fused cell, which is surrounded initially by the residual spermatocyte/spermatid cytoplasm. Injecting activated instead of metaphase-arrested oocytes during ROSI and/or removing the spermatid cytoplasm prior to microinjection may provide a developmental advantage by skipping the unnecessary ''reprogramming'' step that may cause the asynchrony between the development of the intrinsic female pronucleus and the round spermatid-derived male pronucleus. Gene imprinting is presumably completed at or even before the round spermatid stage (Kimura and Yanagimachi, 1995a). Primary (Ogura *et al.,* 1997; Sasagawa *et al.,* 1998) and secondary (Kimura and Yanagimachi, 1995b) spermatocytes were used to obtain viable offspring in mice.

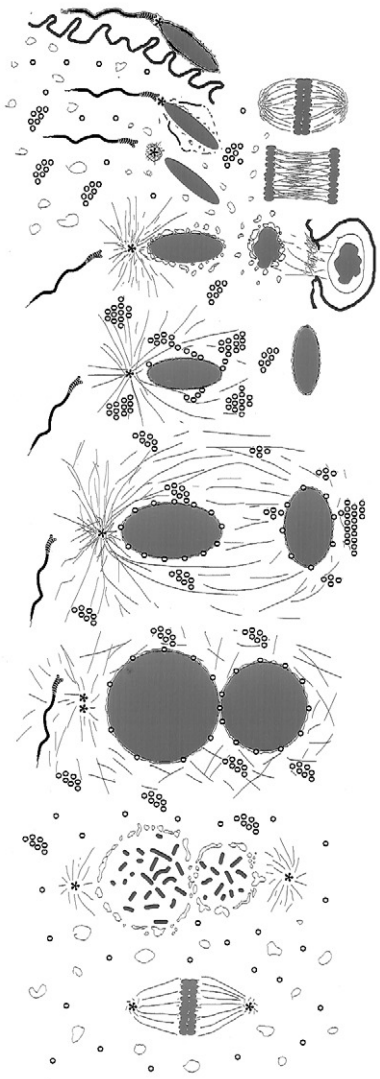
Besides the timing of microinjection, the selection of an appropriate spermatogenic cell is crucial for the developmental success and unbiased interpretation of the results of ROSI. Round spermatids can be confused easily for other spermatogenic or somatic cell types in testicular cell preparations (Sousa *et al.,* 1998). A simple, noninvasive and unambiguous method for the selection of round spermatids is sought by both clinicians and researchers. Methods based on gravity sedimentation (Lam *et al.,* 1970; Grabske *et al.,* 1975; Romrel *et al.,* 1976), percoll separation (Bucci *et al.,* 1986), and cell sorting (Aslam *et al.,* 1998; Gledhill *et al.,* 1990) work well wherever large quantities of testicular tissue can be obtained. These methods, however, cannot be applied to the diagnostic and clinical treatment of human subjects limited by a minute size of tissue samples obtained by testicular biopsy. On-stage selection methods are needed to single out the individual round spermatids in such programs. The presence of an acrosomal granule, as revealed by phase-contrast or differential interference contrast microscopy, remains the main criterion for the on-stage selection of round spermatids (Tesarik and Mendoza, 1996; Sofikitis *et al.,* 1997; Verheyen *et al.,* 1998; Yamanaka *et al.,* 1997). The effectiveness of such selection is relatively low and requires substantial experience. Testsimplets, a staining kit composed of *N*-methylene blue and cresyl violet acetate, was used to identify round spermatids in human testicular biopsies (Angelopoulos *et al.,* 1997). DNA-binding fluorescent dyes Hoechst 33342 and ethidium bromide were used for round spermatid selection based on the estimations of their DNA content (Reyes *et al.,* 1997). We have developed a noninvasive method for the on-stage selection of individual round spermatids based on the polarized patterns of mitochondria (see Fig. 8A) in these cells, as vis-

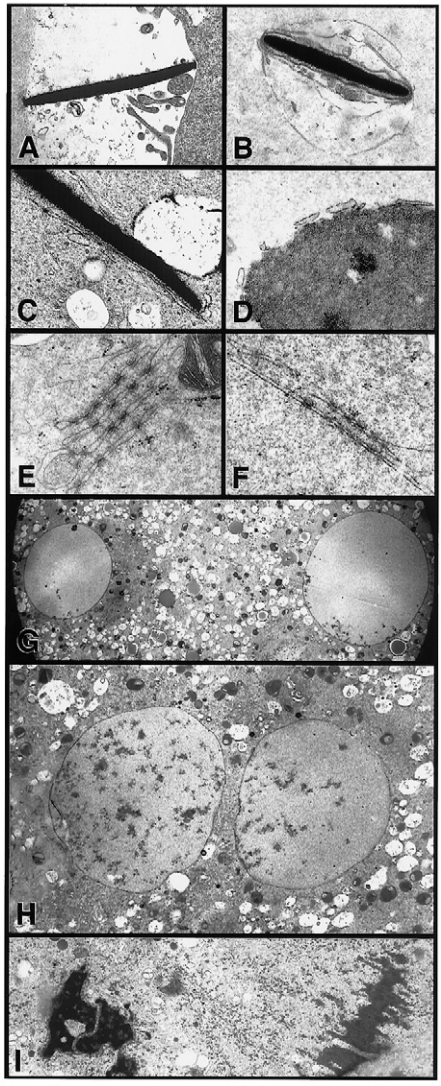
ualized by epifluorescence microscopy after their incubation with vital, mitochondrion-specific fluorescent probes, MitoTracker CMTMRos or MitoTracker Green FM (P. Sutovsky *et al.,* in preparation). The advantage of such a method over the other cytological stains is that MitoTracker dyes, which bind to the spermatid mitochondria presumably doomed for destruction in oocyte cytoplasm, may be less harmful than the DNA-binding dyes used in those other protocols. Ultraviolet photon excitation of blue fluorescent dyes may cause cellular damage, especially if the dyes are bound directly to DNA (Simerly and Schatten, 1993).

3. Nuclear Transfer and Cloning

Despite the enormous progress of cloning and nuclear transfer achieved since the mid-1990s (Campbell *et al.,* 1996; Cibelli *et al.,* 1998; Kato *et al.,* 1998; Vignon *et al.,* 1998; Wakayama *et al.,* 1998; Wilmut *et al.,* 1997), the major drawback of these methods, i.e., the poor fitness and low survival rates of cloned offspring, is yet to be addressed by focused research. The possibility that the nuclear transfer procedure may contribute to the high incidence of ''large offspring syndrome'' in farm animals has been discussed (Young *et al.,* 1998), yet other factors such as *in vitro* embryo culture, imbalanced maternal diets, and asynchronous embryo transfer may be responsible. Avoiding the inheritance of foreign mtDNA and heteroplasmy may also improve the fitness of cloned animals (reviewed extensively in Section VI,D). Evidence is accumulating that suggests the existence of heteroplasmy in animals produced by nuclear transfer (Meirelles *et al.,*

FIG. 10 Structural events of mammalian fertilization. The fertilizing spermatozoon binds to and fuses with the oolemma (A) and is deprived of its perinuclear theca and nuclear envelope (NE) during its incorporation into oocyte cytoplasm (B). Following sperm incorporation, membrane vesicles gather around the sperm chromatin and fuse on its surface into a continuous nuclear envelope, which at this stage lack nuclear pore complexes (C). Likewise, the maternal chromatin becomes separated from the cytoplasm by a nuclear envelope formed by the fusion of membrane vesicles on the surface of decondensing chromatin (D). Concomitant with the formation of the pronuclear NEs, the sperm-borne centriole (asterisks in the drawing) is released into the oocyte cytoplasm and forms sperm aster microtubules using oocyte-produced tubulin molecules. Annulate lamellae (E), the cytoplasmic stacks of nuclear pore complexes, are formed in activated oocytes and are eventually recruited to the pronuclear region. Insertion of nuclear pores into the nuclear envelopes of male and female pronuclei (F) provides a channel for nucleocytoplasmic transport that promotes the developments of male and female pronuclei (G). The full-size pronuclei eventually become apposed (H) by the action of sperm aster microtubules. After reaching the apposition and completing DNA replication, the chromatin in both pronuclei recondenses, the nuclear envelopes disappear, and the zygote enters first mitosis (I). Reprinted with permission from Sutovsky and Schatten (1998).

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1999; Plante *et al.,* 1992; Smith *et al.,* 1991). Follow-up studies of cloned animals, including tissue analysis and metabolic tests, should be done to determine the effects of cloning on the fitness and survival rates after the nuclear transfer of blastocysts and somatic cells. In addition to mtDNA, foreign mRNA can be carried over into the zygotes produced by nuclear transfer and translated in their cytoplasm (Parry and Prather, 1995).

V. Concluding Remarks

Data summarized in this review reveal an intriguing complementarity of sperm–oocyte union at fertilization (Fig. 10). Both gametes carry a complementary arsenal of organelles and molecules that can promote normal development only if they are combined. The rejection of most organelles in the residual cytoplasmic droplet allows for the sperm motility necessary to reach the oocyte. At the same time, cytoplasmic pools of the oocyte store organelles and molecules missing from the spermatozoa. Therefore, sperm and oocytes work in a key and lock-like fashion during fertilization, when the stripped-down spermatozoon unlocks the developmental potential of the richly endowed oocyte. Besides the contribution of nuclear DNA, the paternal inheritance of the sperm centriole for the biparentally contributed reconstitution of the zygotic centrosome is well documented in several nonrodent mammalian species. Sperm-contributed cytosolic oscillogens are responsible for oocyte activation at fertilization and may be introduced into oocyte cytoplasm with the sperm perinuclear theca. Paternal mitochondria and mtDNA, although eliminated by a species-specific mechanism during natural fertilization, can be carried over into oocyte cytoplasm and propagated further by new assisted fertilization protocols that replace the fertilizing mature spermatozoon by immature germ cells, embryonic blastocysts, or somatic cell nuclei. The microtubule cytoskeleton of the zygote, although organized by the sperm centriole, is essentially derived from the maternal pools of tubulins and centrosomal proteins. Similarly, the components of the nuclear envelope, nuclear pore complexes, and the nuclear matrix are maternally derived. Most other sperm accessory structures, including fibrous sheaths, axonemal microtubule doublets, and outer dense fibers, appear to be destroyed after fertilization. The question of whether the sperm plasma membrane becomes incorporated into zygotic plasma membrane at fertilization remains unresolved.

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Coat Proteins Regulating Membrane Traffic

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This review focuses on the roles of coat proteins in regulating the membrane traffic of eukaryotic cells. Coat proteins are recruited to the donor organelle membrane from a cytosolic pool by specific small GTP-binding proteins and are required for the budding of coated vesicles. This review first describes the four types of coat complexes that have been characterized so far: clathrin and its adaptors, the adaptor-related AP-3 complex, COPI, and COPII. It then discusses the ascribed functions of coat proteins in vesicular transport, including the physical deformation of the membrane into a bud, the selection of cargo, and the targeting of the budded vesicle. It also mentions how the coat proteins may function in an alternative model for transport, namely via tubular connections, and how traffic is regulated. Finally, this review outlines the evidence that related coat proteins may regulate other steps of membrane traffic.
KEY WORDS: Coated vesicle transport, Secretion, Endocytosis, COPI, COPII, Clathrin, Adaptors, AP-3.

I. Introduction

The hallmark of eukaryotic cell organization is compartmentalization, the generation and maintenance of organelles of different protein and lipid compositions in order to optimize the biochemical reactions that occur within and on them. Proteins destined for intracellular organelles (other than mitochondria and chloroplasts), for the plasma membrane, or for secretion are synthesized with an N-terminal signal sequence, which interacts with the signal recognition particle to ensure their translocation into

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the endoplasmic reticulum (ER) lumen (Johnson, 1997). Here the proteins undergo quality control; they are acted on by numerous chaperones and often also glycosylation enzymes that enable proper folding, and oligomerization, if necessary, into a mature structure (Hammond and Helenius, 1995). Misfolded proteins are retrotranslocated to the cytosol, where they are ubiquitinated and sent to the proteosomes for degradation (Brodsky and McCracken, 1997). Correctly folded proteins are either retained in the ER if this is their home compartment or transported further along the secretory pathway. This is highly dependent on membrane traffic, which must be tightly regulated to ensure that proteins and lipids are not only delivered to the right compartment, but also retained there, recycled, or transported further as necessary. The most popular view of membrane traffic is the vesicle shuttle model, whereby small coated vesicles bud from a stable donor compartment and transport selected cargo to a stable acceptor compartment, with which they fuse after uncoating. Vesicles may travel in both anterograde and retrograde directions. A variation on this model is the maturation model, in which the compartments are viewed not as stable, but as constantly maturing as a result of continuous removal and addition of material by retrogradely moving vesicles. The third model is the tubular transport model, in which coat proteins are proposed to play a rather different role. Because the vesicle shuttle model is the most widely accepted of the three, the major focus of this review will be on the role of the coat proteins in vesicular transport, with a relatively brief discussion of their potential roles in the other models later on.

The outline of a round of vesicular transport is briefly as follows: coat proteins are recruited from a cytosolic pool to specialized sites on the donor membrane, usually on activation of a specific small GTP-binding protein by its donor membrane-bound GTP exchange factor. This leads to the formation of a coated bud, into which cargo and vesicle-specific v-SNAREs are packaged and concentrated before the bud pinches off to form a coated vesicle. The vesicle is transported to the donor membrane, usually by diffusion over short distances, but is also facilitated by motor transport along cytoskeletal filaments for long distances. GTP hydrolysis on the small GTP-binding protein then renders the coat unstable (although additional proteins are necessary for this in some cases), leading to uncoating. This allows the vesicles to dock through binding of the v-SNAREs to their cognate t-SNAREs, which are specific for the correct target membrane and thus act as vesicle receptors. Rab proteins, after recruitment to the donor membrane or transport vesicle, undergo a GTP-controlled cycle to regulate the assembly of the SNARE complex and thus control the timing of vesicle docking at the target membrane (Hay and Scheller, 1997), although other roles for rabs are also emerging (Martinez and Goud, 1998). The SNARE complex is required for fusion, which does not involve the coat proteins

and so will not be further discussed here, but see Haas (1998) and Hanson *et al.* (1997) for reviews. The released cargo is then either retained in the target compartment or sorted into new vesicles for transport to the next compartment, whereas the v-SNAREs, recycling receptors, and any escaped proteins will be packaged for return to the original donor compartment, not necessarily by the same coats.

The first coat to be identified on a vesicle was the clathrin coat in the endocytic pathway (Roth and Porter, 1964; Kanaseki and Kadota, 1969). It has since become apparent that despite their variations in structure and subunit composition, the general mechanism of action of the clathrin, COPI, COPII, and AP-3 coats is very similar. We will discuss each coat complex in turn in the context of vesicular transport.

II. Clathrin and Adaptor Proteins

A. Clathrin-Mediated Endocytosis

1. History

Eukaryotic cells use alternative pathways to endocytose substances from the external environment and internalize their plasma membrane (Mellman, 1996), the best characterized being clathrin-dependent endocytosis. Roth and Porter (1964) first described clathrin-coated vesicles as being involved in the uptake of yolk proteins in the mosquito oocyte. They observed that pits invaginate into the cell and pinch off from the plasma membrane to form vesicles coated on their cytosolic surface with densely packed T-like projections. They proposed that these vesicles then lose their coat and fuse to form small crystalline yolk droplets. Since then, many other studies have confirmed this general sequence of events in the endocytic cycle and have shown that endocytosis by clathrin is receptor mediated, meaning that cargo molecules bind to specific transmembrane receptors, accumulate in coated pits, and enter the cells as ligand–receptor complexes in clathrin-coated vesicles (Kornfeld and Mellman, 1989; Gruenberg and Maxfield, 1995). The clathrin coat is recruited to different membranes by specific adaptor proteins (APs). The AP-2 adaptor recruits clathrin to the plasma membrane to mediate endocytosis. These clathrin-coated vesicles deliver receptor–ligand complexes and solutes to early endosomes, where receptors and ligands are either recycled to the plasma membrane or transported to the lysosomes to be degraded. Similar events occur in the biosynthetic pathway: a different adaptor, AP-1, leads to clathrin-coated vesicle formation at the *trans*-Golgi network (TGN) and these vesicles transfer newly synthesized lysosomal

proteins bound to mannose-6-phosphate receptors to endosomes, (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). AP-1 and clathrin have also been implicated in basolateral targeting from the endosome (Futter *et al.,* 1998). In addition to promoting transport between specific organelles in the cell, one of the first functions ascribed to clathrin-coated vesicles was the maintenance of the proper membrane balance, in particular following synaptic vesicle fusion (De Camilli, 1995).

2. Components and Structure of the Clathrin Coat

Clathrin-coated vesicles can be distinguished very easily from the other types of coated vesicles, COPI and COPII (see later), by the presence on their surface of the characteristic lattice-like or basket-like structures first described by Kanaseki and Kadota (1969). These lattices consist of 12 pentagonal units plus a variable number of hexagonal units, depending on the size of the coated vesicle, which can vary from 50 to 250 nm in diameter.

Biochemical analyses have revealed that the coat of clathrin-coated vesicles consists of two major complexes: the clathrin complex and the adaptor complex (Pearse, 1975, 1976; Robinson, 1994). The basic unit of the clathrin complex, the triskelion, is composed of three clathrin heavy chains and three clathrin light chains. The adaptor complex consists of two ~100-kDa proteins (the adaptins), a ~50-kDa medium chain (μ), and a ~20-kDa small chain (σ) (see Table I). It consists of a central brick-like

TABLE I
AP Subunits in Mammals

Subunit	Size (kDa)	Reference
AP-1		
γ	108	Robinson (1990)
β1	105	Kirchhausen *et al.* (1989)
μ1 (AP47)	47	Nakayama *et al.* (1991)
σ1 (AP19)	19	Kirchhausen *et al.* (1991)
AP-2		
α	92	Robinson (1989)
β2	105	Kirchhausen *et al.* (1989)
μ2 (AP50)	50	Thurieau *et al.* (1988)
σ2 (AP17)	17	Kirchhausen *et al.* (1991)
AP-3		
δ	125	Simpson *et al.* (1997)
β3	121	Simpson *et al.* (1997); Newman *et al.* (1995); Dell'Angelica *et al.* (1997)
μ3	47	Pevsner *et al.* (1994)
σ3	22	Simpson *et al.* (1997); Dell'Angelica *et al.* (1997)

structure (the ''head''), composed of the bulk of adaptins plus the medium and the small chains, and two out-jutting appendages or ''ears,'' which correspond to the carboxy-terminal domains of the two adaptins (Heuser and Keen, 1988). Three different adaptor complexes, AP-1, AP-2, and AP-3, have been described so far and characterization of at least a fourth one is underway. The AP-2 adaptor is localized to plasma membrane-coated pits, whereas AP-1 is associated with vesicles budding from the TGN (Robinson, 1994, 1997). AP-2 is composed of α- and β2-adaptins, the medium chain μ2 (AP50), and the small chain σ2 (AP17). The AP-1 complex consists of γ- and β1-adaptin associated with μ1 (AP47) and σ1 (AP19) chains. The more recently identified AP-3 complex (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997; Cowles *et al.,* 1997a) is described in more detail in the AP-3 section, as its association with clathrin is currently controversial (Simpson *et al.,* 1996; Dell' Angelica *et al.,* 1998).

3. Clathrin Coat Assembly

It has long been known that, *in vitro,* clathrin alone or in combination with adaptors can assemble into empty coats or cages, which produce the characteristic lattice of coated vesicles (Pearse and Crowther, 1987). However, the formation of coated vesicles only takes place at specialized sites of the plasma membrane, where concentration of a subset of receptors occurs (Anderson *et al.,* 1976; Goldstein *et al.,* 1979). This suggests that interactions between coat subunits and specific membrane proteins must occur to achieve both cargo specificity and bud site selection.

Adaptors were first identified as factors promoting clathrin assembly into cages (Keen *et al.,* 1979; Keen, 1987; Pearse and Robinson, 1984) and are responsible for clathrin recruitment to the membrane (Robinson, 1994), thus the first step of clathrin-coated vesicle formation is thought to be the recruitment of adaptors from the cytosol to the membrane. Work with systems *in vitro* (Robinson and Kreis, 1992; Seaman *et al.,* 1993; Chang *et al.,* 1993; Traub *et al.,* 1993) has shown that AP-2 and AP-1 adaptors are able to associate specifically with the plasma membrane or TGN, respectively, but it is unclear how this occurs. There is strong evidence that the cytoplasmic tails of membrane proteins can bind to adaptors and are involved in their specific recruitment. In other words, cargo sorting and clathrin-coated vesicle formation may be coupled processes, as discussed in more detail in the section on cargo sorting. The adaptors have only a low affinity for the cargo-sorting signals, however, so it is likely that factors other than cargo are involved in adaptor recruitment, as discussed next.

a. AP-1 Recruitment Factors

i. Cytosolic Factors Parallels have been observed between the recruitment of the AP-1 adaptor and the COPI precursor, coatomer, to Golgi

membranes. Both processes are blocked by brefeldin A (BFA) and enhanced by GTPγS (Wong and Brodsky, 1992; Robinson and Kreis, 1992; Donaldson *et al.,* 1990), suggesting that, like the coatomer, AP-1 is recruited to the membrane through activation of a specific small GTP-binding protein. This is most likely to be ADP-ribosylation factor 1 (ARF1), as *in vitro* AP-1 is poorly recruited to Golgi membranes in the presence of ARF-depleted cytosol and the addition of recombinant ARF1 restores the binding (Stamnes and Rothman, 1993).

ii. Membrane-Associated Factors. The targeting determinants for the AP-1 adaptors themselves are contained in the N and C termini of γ-adaptin and also in the small and medium chains (Page and Robinson, 1995; Robinson, 1993). The two-hybrid system and cross-linking experiments have been used to identify the membrane-bound components responsible for adaptor targeting to the correct membrane (Seaman *et al.,* 1996). Three novel proteins have been found to interact specifically with AP-1: p75 interacts with γ-adaptin, p60 with μ1, and p80 with β1 adaptin. p80 has been identified independently as binding AP-1 adaptors by affinity chromatography (Mallet and Brodsky, 1996). These proteins interact with the targeting determinant-containing subunits of the adaptor complex, suggesting that these proteins, especially p60 and p75, belong to a putative docking complex. Attempts to identify the nature of these novel proteins have been unsuccessful so far.

b. AP-2 Recruitment Factors Targeting determinants for the AP-2 adaptor itself are contained in the N and C termini of α-adaptin and also in the small and medium chains (Page and Robinson, 1995; Robinson, 1993). Despite the high homology between AP-1 and AP-2 adaptors, they do not employ the same mechanisms for recruitment to membranes (West *et al.,* 1997). Unlike AP-1 and AP-3, recruitment of AP-2 to the donor membrane is cytosol independent (Smythe *et al.,* 1992) and insensitive to BFA and GTPγS, suggesting that ARF1 is not involved in this process (Seaman *et al.,* 1993; Robinson and Kreis, 1992; Hunziker *et al.,* 1992). However, GTPγS can cause mistargeting of AP-2 from the plasma membrane to late endosomes (Seaman *et al.,* 1993), implying that another small GTP-binding protein may be involved (Carter *et al.,* 1993). A potential candidate is ARF6, whose localization is restricted to the plasma membrane and is BFA insensitive (Cavenagh *et al.,* 1996; Peters *et al.,* 1995). In addition, overexpression of ARF6-GTP (Q67L), a mutant of ARF6 defective in GTP hydrolysis, decreases the rate of transferrin uptake (D'Souza-Schorey *et al.,* 1995). However, the failure to detect ARF6 in clathrin-coated pits or vesicles (Cavenagh *et al.,* 1996) is inconsistent with a role of ARF6 in clathrin coat formation. A member of another class of small GTP-binding proteins, Rab5, has been localized to the plasma membrane, clathrin-coated

vesicles, and endosomes (Bucci *et al.,* 1992; Chavrier *et al.,* 1990) and has been proposed to act as regulator of fusion of clathrin-coated vesicles with early endosomes (Bucci *et al.,* 1992). However, its participation in coated vesicle formation remains to be determined.

AP-2-binding sites at the plasma membrane were first reported in human fibroblasts (Anderson, 1993; Mahaffey *et al.,* 1990) and later attributed to the plasma membrane-associated protein synaptotagmin in both neuronal and nonneuronal cells (Zhang *et al.,* 1994b; Li *et al.,* 1995a). In *Caenorhabditis elegans* synaptotagmin mutants, synaptic vesicles are depleted at synaptic terminals due to the insufficient recycling of synaptic vesicles from the plasma membrane (Jorgensen *et al.,* 1995), although it is not clear whether this is a direct consequence of the lack of AP-2 binding.

A novel protein, Eps15, has been found associated with AP-2 and endocytic pits (Benmerah *et al.,* 1995, 1996; Tebar *et al.,* 1996; van Delft *et al.,* 1997). Identified as a substrate of the EGF receptor tyrosine kinase (Fazioli et al., 1993), Eps15 was initially proposed to have a role in signal transduction, but its association with α-adaptin (Benmerah *et al.,* 1996) and the inhibition of EGF and transferrin internalization by anti-Eps15 antibodies (Carbone *et al.,* 1997) strongly suggest a function in receptor-mediated endocytosis. Electron microscopy has revealed that Eps15 is mainly associated with membrane-bound AP-2, at the rims of endocytic coated pits, but not in clathrin-coated vesicles, which correlates with the observed dissociation of Eps15 from AP-2 during clathrin coat formation (Cupers *et al.,* 1998). The amino terminus of Eps15 contains three conserved repeats of 70 amino acids, referred to as EH domains (for Eps15 homology), which have also been identified in several other proteins, including the Eps15-related protein Eps15R (Wong *et al.,* 1995); End3p and the yeast homolog of Eps15, Pan1p, both required for endocytosis of α-factor (Benedetti *et al.,* 1994; Wendland *et al.,* 1996); and synaptojanin, which may play a role in synaptic vesicle recycling (McPherson *et al.,* 1996). The role of this EH motif is not known, but it may be involved in protein–protein interactions (Wong *et al.,* 1995; Salcini *et al.,* 1997), and the interactions of Pan1p with members of the clathrin assembly protein (AP180) family (Ahle and Ungewickell, 1986) and synaptojanin-like proteins have led to the proposal that Pan1p/Eps15 acts as a coordinator of the early steps of endocytosis by bringing together endocytic and actin-cytoskeleton organizing proteins (Di Fiore *et al.,* 1997; Wendland and Emr, 1998).

c. Clathrin Recruitment After adaptor binding, the next step in the clathrin-coated vesicle cycle is the recruitment of clathrin. *In vitro,* clathrin triskelions can be released from coated vesicles by adding high concentrations of Tris and are able to self-assemble and form baskets with a structure similar to that seen on the surface of coated vesicles (Keen *et al.,* 1979;

Keen, 1987; Pearse and Crowther, 1987). Although adaptor proteins are not necessary for this, they do facilitate assembly *in vitro* and play a critical role in clathrin recruitment from the cytosol *in vivo* (Chang *et al.,* 1993; Robinson, 1994). Clathrin-binding sites have been identified in the appendage domains of both β1 and β2, and also β3 of the AP-3 coat (Gallusser and Kirchhausen, 1993; Shih *et al.,* 1995; Traub *et al.,* 1995; Dell'Angelica *et al.,* 1998).

Cytosolic clathrin is associated with two other proteins, the heat shock protein Hsc70 (or uncoating ATPase; Schmid *et al.,* 1984) and the valosin-containing protein, VCP (Pleasure *et al.,* 1993; Chappell *et al.,* 1986). This might explain why cytosolic pools of clathrin and adaptors do not interact with each other. *In vitro,* Hsc70 has been shown to release clathrin from the coated vesicles, although it remains to be shown if this is the case *in vivo* (Schmid *et al.,* 1984). The 100K Dna-J related protein, auxilin, acts as a cofactor for Hsc70 by recruiting it to clathrin lattices in the presence of ATP and promoting its ATPase activity (Ungewickell *et al.,* 1995). Interestingly, sequence analyses have revealed that VCP is a member of the triple A family of ATPases and is identical to an NSF-like ATPase, p97, which has been proposed to mediate cisternal regrowth from mitotic Golgi fragments in concert with NSF (Pfeffer, 1996; Rabouille *et al.,* 1995). The role of VCP in the clathrin-coated vesicle cycle is not clear, but its similarity to chaperonins indicates that VCP may modulate the interaction of clathrin with other proteins. Thus, Hsc70 and VCP may prevent clathrin from forming nonspecific interactions.

Another type of protein that interacts with clathrin is arrestin. Arrestin-3 and β3-arrestin bind the β_2-adrenergic receptor, a G-protein-coupled receptor, on agonist activation (Goodman *et al.,* 1996). Moreover, arrestin binds directly to clathrin with high affinity and may act as an adaptor between the receptor and clathrin, with these sequential interactions leading to receptor internalization (Goodman *et al.,* 1996). However, arrestins do not induce coat assembly (Goodman *et al.,* 1997), suggesting that AP-2 is still required for the recruitment of this receptor into coated pits.

Genetic studies in yeast have provided another approach to studying the function of clathrin. In some cases, deletion of the clathrin heavy chain is lethal (Lemmon and Jones, 1987), whereas in others, fluid-phase endocytosis and transport to vacuoles are only reduced partially (~50%), with secretion being unaffected (Payne and Schekman, 1985; Payne *et al.,* 1987, 1988). This is strong evidence for the existence of clathrin-independent endocytosis, which occurs in many cell types (Sandvig and van Deurs, 1991, 1994; Lamaze and Schmid, 1995), the best described being caveolae-mediated endocytosis (Parton, 1996). However, no well-characterized membrane proteins have been shown to use the clathrin-independent pathway, and it is still a matter of debate as to its parallel existence with the clathrin-dependent

pathway, as the former is only clearly observed when the latter is impaired. Clathrin coat recruitment is insufficient for vesicle budding *in vivo*. Invagination, constriction, and scission steps, which involve additional proteins and also ATP and GTP hydrolysis, are required for vesicle production. Whereas no ATPases have been identified yet, dynamin has emerged as a strong candidate for the GTP-hydrolyzing protein (Schmid and Damke, 1995) and is discussed in more detail in Section V.

4. Role of Clathrin-Coated Vesicles in Synaptic Vesicle Recycling

During nerve stimulation the pool of synaptic vesicles is depleted rapidly, leading to an increase in the surface area of the synaptic membrane. After exocytosis, this excess membrane is internalized rapidly and reused for the formation of new synaptic vesicles (Heuser and Reese, 1973). Two distinct recycling pathways have been shown to coexist (Koening and Ikeda, 1996; Takei *et al.,* 1996). The first involves the generation of synaptic vesicles through endosomal intermediates, and the second generates recycling vesicles directly from the plasma membrane. The endosomal intermediates are thought to be derived from plasma membrane clathrin-coated invaginations (Heuser and Reese, 1973; Ceccarelli *et al.,* 1973; Takei *et al.,* 1996). Although it is not clear whether the synaptic vesicles formed from this endosomal compartment are derived from coated vesicles (Koenig and Ikeda, 1996), several lines of evidence indicate that this may be the case. In nerve terminal membranes incubated with GTPγS, clathrin-coated bud intermediates can be seen on the endosomal compartment in association with AP-2, which lead to the proposal that clathrin-coated vesicles budding from the endosomal compartment are intermediates in synaptic vesicle formation (Takei *et al.,* 1996). However, this has yet to be reconciled with the apparent lack of requirement for clathrin in the recent reconstitution of this budding event with the purified AP-3 complex and ARF-GTP (Faúndez *et al.,* 1998).

The second recycling pathway involves the formation of synaptic vesicles directly from the plasma membrane. The ''kiss-and-run'' hypothesis postulates that the recycling of vesicles does not require intermediate structures such as coated vesicles (Ceccarelli *et al.,* 1973; Fesce *et al.,* 1994). An alternative model, proposed by de Camilli and colleagues, is that recycling vesicles may be generated by clathrin-mediated budding from the plasma membrane (Takei *et al.,* 1996).

B. AP-3

A third adaptor-related protein complex, AP-3, has been identified in both mammals and yeast (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997; Cowles

et al., 1997a). Like AP-1 and AP-2, the AP-3 adaptor is a heterotetramer, which consists of β3, δ, μ3, and σ3 subunits (see Table I).

1. Components of AP-3 Adaptor

The first AP-3 adaptor subunit to be found was the neuron-specific β3B (originally called β-NAP), which was identified as the antigen responsible for autoimmune cerebellar degeneration and was only linked to coat proteins by virtue of its 28% identity with β1 and β2 adaptins (Newman *et al.,* 1995). Immunoprecipitation of brain cytosol with anti-β-NAP antibodies showed that the other proteins in the complex were p160, p47, and p25 (Simpson *et al.,* 1996), the sizes of which suggested that p160 may be the α- and γ-adaptin homolog (δ) of this new complex and p25 (σ3) the small chain homolog of σ1 and σ2. These proteins were indeed identified, along with a ubiquitous form of β-NAP (β3A), by searching the EST database with the respective homologs (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997). p47 (μ3A) turned out to have been identified previously as ray electric organ and rat brain homologs (p47A and p47B) of the adaptor medium chains μ1 and μ2 (Pevsner *et al.,* 1994).

The δ subunit is the probable mammalian homolog (with 24% identity) of the *Drosophila garnet* gene product (Simpson *et al.,* 1997), which is responsible for eye color pigmentation. It has a predicted size of 125 kDa and, despite having only 15% identity to α- and γ-adaptins, mainly restricted to the N-terminal domain, it shares their overall domain structure (an N-terminal trunk and a C-terminal ear, separated by a highly hydrophilic hinge) and contains the WIIGEY consensus sequence found in the α-, β-, and γ-subunits and in the β-COP subunit of coatomer (Robinson, 1989; Ponnambalam *et al.,* 1990; Kirchhausen *et al.,* 1989; Newman *et al.,* 1995; Duden *et al.,* 1991). Two homologs of the adaptor small chains σ1 and σ2 were identified as σ3A and σ3B (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997).

Northern blot analyses revealed that δ, σ3, β3A, μ3A, σ3A, and σ3B are all expressed ubiquitously (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997; Pevsner *et al.,* 1994), with β3B and μ3B being neuron-specific isoforms of β3A and μ3A, respectively (Newman *et al.,* 1995; Pevsner *et al.,* 1994). AP-3 seems to have a tissue-specific subunit composition, although no neuron-specific isoforms of either δ or σ3 have been reported so far.

The endogenous AP-3 complex is associated with perinuclear structures, partially colocalizing with the TGN, and more peripheral punctate structures that colocalize with transferrin–HRP-positive endosomal tubules (Simpson *et al.,* 1996, 1997; Dell'Angelica *et al.,* 1998). Despite the overall similarity in pattern, no colocalization of AP-3 proteins was observed with either TGN-associated AP-1 (γ-adaptin) or p200/myosin, a TGN-associated

protein that had been proposed to be a component of a nonclathrin coat (Narula *et al.,* 1992; Narula and Stow, 1995). Whether AP-3 associates with clathrin, like AP-1 and AP-2, is controversial. Although AP-3 has been found associated with clathrin, both by electron microscopy and *in vitro*-binding studies (Dell'Angelica *et al.,* 1998), this contradicts earlier electron microscopy studies and the absence of the AP-3 complex in clathrin-coated vesicle preparations (Simpson *et al.,* 1996; Newman *et al.,* 1995). Recruitment of the purified AP-3 complex to endosomes requires ARF1, explaining the BFA sensitivity of this coat, but budding appears to be able to occur independently of clathrin (Faúndez *et al.,* 1998; Ooi *et al.,* 1998). Furthermore, in yeast the AP-3 cargo alkaline phosphatase (see later) is missorted dramatically in AP-3 mutants, but not in clathrin heavy chain mutants (Seeger and Payne, 1992).

2. AP-3 Function

Several lines of evidence suggest that the AP-3 adaptor may be involved in the transport to lysosomes. First, like μ1 and μ2, μ3 is able to interact with the tyrosine-based motifs (Dell'Angelica *et al.,* 1997) that act as sorting signals (Ohno *et al.,* 1995), suggesting that the AP-3 complex may be involved in sorting proteins bearing such motifs. Furthermore, mutants of the *Drosophila garnet* gene product (δ homolog) exhibit impaired eye pigmentation, probably resulting from a defect in pigment granule biogenesis (Simpson *et al.,* 1997). In mammals, the pigment granule equivalents are melanosomes, which are lysosome-like structures, and patients with Chediak–Higasi syndrome (related to the *beige* pigment mutation in mice) present defects in lysosome biogenesis (Burkhardt *et al.,* 1993). More direct evidence in mammalian cells is that a 60% reduction in μ3A expression levels by antisense oligonucleotides results in partial misrouting of two lysosomal proteins, LIMP-II and LAMP-I, to the plasma membrane, presumably from the TGN (le Borgne *et al.,* 1998).

The strongest evidence for AP-3 function in protein delivery to lysosomes, however, came, with the identification of the yeast AP-3 complex in a screen for factors involved in the transport of alkaline phosphatase in a novel pathway from the Golgi to the vacuole (Cowles *et al.,* 1997a; Panek *et al.,* 1997; Stepp *et al.,* 1997). Apl6p and Apl5p were identified as the yeast counterparts of β3A and δ, respectively, and the other subunits were found by searching the yeast database for homologs of μ3 (Apm3p) and σ3 (Aps3p). Deletion of these proteins prevents the appropriate localization of alkaline phosphatase and Vam3p (a vacuolar t-SNARE) to the vacuole, but has no effect on the vacuolar delivery of carboxypeptidases Y and S, which are known to use the classical pathway. Thus the study of yeast

mutants has allowed parallels in AP-3 function to be drawn between yeast and mammals.

Whereas the ubiquitous form of AP-3 is therefore involved in transport between the TGN and lysosomes, the nerve-specific form of the AP-3 coat complex may have another function, originally proposed by Kelly and collaborators: that of synaptic vesicle formation from endosomes (Faúndez *et al.,* 1998). They were able to reconstitute synaptic vesicle budding *in vitro* from PC12 endosomes with just AP-3 and ARF1-GTP. Support for a dual role for AP-3 is that the *mocha* mouse, mutant in the δ subunit common to both ubiquitous and nerve-specific AP-3 complexes, exhibits defects not only in pigmentation and lysosomal function, but also in neurological function (Kantheti *et al.,* 1998). Thus, like COPI (later), AP-3 may be involved in vesicle formation from more than one organelle.

III. Coat Proteins

A. COPI

1. History

COPI was first identified as a 10-nm-thick nonclathrin coat on 70- to 75-nm vesicles formed on adding cytosol and ATP to Golgi membranes in an *in vitro* assay established by Rothman and colleagues. They measured the modification of VSV-G as a result of its presumed transfer from a glycosyltransferase-deficient Golgi cisterna to another VSV-G-free glycosyltransferase-containing one (Balch *et al.,* 1984; Orci *et al.,* 1986, 1989). The observation that GTPγS prevented the uncoating of COPI-coated vesicles (Melancçon *et al.,* 1987) led to their isolation (Malhotra *et al.,* 1989) and biochemical characterization. The coat consists of coatomer (Waters *et al.,* 1991; Stenbeck *et al.,* 1993) and ARF (Serafini *et al.,* 1991), and these and nucleotide triphosphate are sufficient for bud formation from Golgi membranes (Orci *et al.,* 1993; Palmer *et al.,* 1993). A fatty acyl coenzyme A, such as palmitoyl CoA, is necessary for membrane fission (periplasmic fusion), allowing vesicle release (Pfanner *et al.,* 1989), but the other components required for this are still unknown. Hydrolysis of GTP on ARF is necessary for uncoating (Tanigawa *et al.,* 1993), permitting the vesicle to fuse with the target membrane and discharge its VSV-G cargo (Ostermann *et al.,* 1993), thus these vesicles are functional for transport. Additional evidence that COPI functions in this assay is that an anti ζ-COP antibody that appears to prevent coatomer from binding to membranes *in vitro* has a partially inhibitory effect (Kuge *et al.,* 1993).

2. Components of the Coatomer

Coatomer (short for coat protomer) is a complex of seven subunits called COPs (coat proteins; Waters *et al.,* 1991), which makes up the COPI coat, perhaps together with the small GTPase ARF, although it is no longer clear if ARF is really a stoichiometric component of the coat (Serafini *et al.,* 1991; Ktistakis *et al.,* 1996; Stamnes *et al.,* 1998). The seven subunits, named α–ζ COP, have all been cloned and sequenced in mammalian cells and their homologs have been identified in yeast (see Table II). All the COPI genes, bar ε-COP, are essential for viability in yeast, illustrating the importance of secretory coat proteins to membrane traffic. In yeast, the α, δ, and ζ subunits were identified as Golgi-ER retrieval mutants (Letourneur *et al.,* 1994; Cosson *et al.,* 1996), whereas β, β' and γ-COP were isolated as secretory mutants (Duden *et al.,* 1994; Harter *et al.,* 1993; Hosobuchi *et al.,* 1992). Unexpectedly, a lethal temperature-sensitive mammalian mutant in ε-COP was discovered before any yeast mutant: a CHO cell line, ldlF, in which ε-COP is degraded at the restrictive temperature, was isolated for its defect in endocytosis, although it was also defective in secretory transport, as expected (Hobbie *et al.,* 1994; Guo *et al.,* 1994, 1996). Yeast ε-COP was identified as a high copy suppressor of a particularly restrictive mutant in α-COP (Duden *et al.,* 1998) and appears to stabilize it. ε-COP may thus have a specific protective function, not only in yeast (Duden *et al.,* 1998), but also in mammalian ldlF cells (Gu *et al.,* 1997; Gomez *et al.,* submitted). The β-, δ-, and ζ-COP subunits are similar in size to and show weak homology to some domains of the β-adaptin, AP47/AP50 ($\mu1/\mu2$) and AP17/AP19 ($\sigma2/\sigma1$) subunits of the clathrin adaptor complexes, respectively, implying that these domains are important for general aspects of coat function (Duden *et al.,* 1991; Kuge *et al.,* 1993; Faulstich *et al.,* 1996; Cosson *et al.,* 1996). Based on its homology to the AP47/$\mu1$ subunit of AP-1, δ-COP has been proposed to be a kinase (Nakayama *et al.,* 1991) and its interactions with multiple subcomplexes of COPs dissociated *in vitro* may mean that it also has a chaperone-like role (Lowe and Kreis, 1995).

3. Sites of Action and Function of COPI

The wealth of information gleaned from Rothman's pioneering transport assay (Balch *et al.,* 1984) strongly implicated COPI in vesicle budding from the Golgi and was interpreted as COPI mediating anterograde transport of VSV-G within the Golgi stack. This was supported by the finding that BFA prevents GDP–GTP exchange on ARF, leading to the rapid dissociation of coatomer from the membrane, prevention of COPI budding *in vitro,* and a block of secretion *in vivo* (Donaldson *et al.,* 1990; Donaldson *et al.,* 1992b; Lippincott-Schwartz *et al.,* 1989; Orci *et al.,* 1991b; Misumi *et al.,*

TABLE II

Coatomer Subunits in Mammals and Their Yeast Homologs

Coatomer subunit	Size (kDa)	Source	Reference	Yeast homolog	Reference	% identity
α	160	Bovine liver Human	Faulstich *et al.* (1996) Chow and Quek (1996)	Ret1p	Letourneur *et al.* (1994) Gerich *et al.* (1995)	46
β	107	Rat liver	Duden *et al.* (1991)	Sec26p	Duden *et al.* (1994)	43
β'	102	Bovine brain Human	Stenbeck *et al.* (1993) Harrison-Lavoie *et al.* (1993)	Sec27p	Duden *et al.* (1994) Harter *et al.* (1993)	46
γ	97	Bovine brain	Harter *et al.* (1996)	Sec21p	Hosobuchi *et al.* (1992)	40
δ	57	Bovine brain Human (archain)	Faulstich *et al.* (1996) Radice *et al.* (1995)	Ret2p	Cosson *et al.* (1996)	35
ε	36	Bovine liver CHO ldlF cells	Hara-Kuge *et al.* (1994) Guo *et al.* (1994)	Sec28p	Duden *et al.* (1998)	33
ζ	20	Bovine liver	Kuge *et al.* (1993)	Ret3p	Cosson *et al.* (1996) Yamazaki *et al.* (1997)	38

1986). However, two problems are that this assay cannot distinguish between the anterograde transport of VSV-G and the retrograde transport of the glycosyltransferase, or even direct fusion between the cisternae, and that the isolated Golgi may be contaminated with other membranes, such as intermediate compartment, which could have given rise to COPI-coated vesicles. Indeed, subsequent data have supported all these alternatives.

First, the yeast secretory mutant Sec21, encoding the γ-subunit of COPI (Hosobuchi *et al.,* 1992), and later also Sec26 (β-COP) and Sec27 (β'-COP) (Duden *et al.,* 1994) were shown to be defective in an earlier step of transport, between the ER and Golgi. Careful electron microscopic analysis then showed that, at least in some cell types, membrane-bound COPI was mostly ($\sim$70%) associated with the *cis*-Golgi and intermediate compartment rather than later Golgi cisternae (Oprins *et al.,* 1993) and colocalized with anterograde VSV-G cargo *en route* to the Golgi (Pind *et al.,* 1994; Griffiths *et al.,* 1995). Direct support for a role of COPI in ER–Golgi transport was that certain anti-β-COP antibodies blocked the ER to Golgi transport of VSV-G (Peter *et al.,* 1993; Pepperkok *et al.,* 1993), similar to the effect of activating (GTP-restricted) ARF mutants (Zhang *et al.,* 1994a; Dascher and Balch, 1994).

The discovery of COPII, shown to mediate ER to Golgi transport of pro-α-factor in yeast in the absence of COPI (Barlowe *et al.,* 1994), upset this model at a time when coatomer had just been shown to bind to the KKXX ER-retrieval signal *in vitro* (Cosson and Letourneur, 1994). This led to the hypothesis that COPI may mediate retrograde transport from the Golgi to the ER, which was supported strongly by the discovery that certain mutations in yeast α-COP (Ret1), β'-COP (Sec27), γ-COP (Sec21), δ-COP (Ret2), and ζ-COP (Ret3) caused defects in the retrieval of KKXX-bearing proteins to the ER, implying that retrograde but not anterograde transport was disrupted (Letourneur *et al.,* 1994; Cosson *et al.,* 1996; Lewis and Pelham, 1996). Coatomer was similarly shown to be necessary for HDEL/KDEL receptor (Erd2p) retrieval in yeast (Lewis and Pelham, 1996), but no cytoplasmic sequence that could interact with the coat has been identified so far. Furthermore, new mutants of yeast γ-COP are defective in ER to Golgi transport of only certain cargo, suggesting that COPI is only indirectly necessary for anterograde transport, perhaps recycling specific cargo receptors of COPII vesicles to the ER (Gaynor and Emr, 1997). In addition, retrograde transport from the Golgi to the ER in yeast was reconstituted *in vitro* and was dependent on the addition of ARF1 and coatomer (Spang and Schekman, 1998).

Given that the role of COPI in retrograde transport cannot be refuted, the question is now whether this is exclusive or if COPI could be involved in both antero- and retrograde ER-to-Golgi traffic. Further support for an anterograde role of COPI includes its localization to certain parts of the

ER under certain conditions (coatomer-rich ER; Orci *et al.,* 1994), although not to the budding zones, and the production of COPI-coated vesicles by adding coatomer and ARF to isolated yeast nuclei, a source of ER, *in vitro* (Bednarek *et al.,* 1995). However, no established cargo was found in such vesicles, and there is no indication that COPI buds from the ER *in vivo.* Furthermore, the ability to bud vesicles from protein-free lipid micelles with just coatomer and ARF-GFP implies that coat assembly is not always specific or physiological (Spang *et al.,* 1998). The strongest evidence that COPI mediates bidirectional transport is the electron microscopic analysis of unperturbed tissue showing that anterograde pro-insulin cargo and the recycling KDEL receptor are present in separate populations of COPI vesicles (Orci *et al.,* 1997), although the destination of the vesicles could not be proven and the study only focused on intra-Golgi transport and need not mean that COPI mediates ER–Golgi anterograde transport too (indeed, the KDEL receptor would be expected to also travel in anterograde vesicles between the ER and Golgi).

If COPI is involved in anterograde transport from the ER to Golgi, there must be a mechanism preventing it from transporting KKXX-bearing ER residents out of the ER. This may be achieved by dilysine-independent mechanisms of retaining such proteins in the ER, as evidenced by the fact that removal of the dilysine motifs only results in slow export (Teasdale and Jackson, 1996), such as binding to a matrix of ER-localized quality control chaperones (Tatu and Helenius, 1997). Alternatively, the dilysine motif could be masked from COPI or there could be different forms of COPI for anterograde and retrograde transport. Very indirect evidence for KKXX masking is that tubulin can bind to this motif *in vitro* and that overexpression of certain KKXX-bearing proteins can lead to a restructuring of the ER into thin tubules that colocalize with microtubules but not COPI (Teasdale and Jackson, 1996). In the case of the IgE receptor, the KKXX-bearing α-subunit is sterically masked by the γ-subunit on oligomerization, thus constituting a quality control mechanism for the retrieval of unassembled complexes to the ER (Letourneur *et al.,* 1995).

Evidence for heterogeneous forms of COPI includes multiple phosphorylation of β- and δ-COPs (Sheff *et al.,* 1996), although no differences in function according to phosphorylation state have been determined yet, and the detection of a form of COPI apparently lacking γ- and δ-COP on endosomes (Whitney *et al.,* 1995; Aniento *et al.,* 1996; see later). An alternative idea is that different parts of the same coatomer somehow bind to distinct cytosolic signals to mediate sorting in different directions, such as $\beta'/\alpha/\varepsilon$ binding to KKXX-like motifs (Lowe and Kreis, 1995; Letourneur *et al.,* 1994; Fiedler *et al.,* 1996) and δ binding to δL motifs (Cosson *et al.,* 1998) for retrograde transport and $\beta/\gamma/\zeta$ binding to phenylalanine motifs for anterograde transport in the secretory pathway (Fiedler *et al.,* 1996).

Support for this model is that coatomer from ldlF ε-COP^{ts} mutant cells cannot bind to KKXX-fusion proteins in the absence of ε-COP, suggesting that ε-COP is important for retrograde cargo selection (Gomez *et al.,* submitted). Preliminary results indicate that this ε-COP-deficient coatomer actually binds instead to FF motifs *in vitro,* perhaps even implicating ε-COP as the directionality switcher (M. Gomez and T.E. Kreis, unpublished results). In addition, the transport of VSV-G to the plasma membrane is severely retarded but not completely inhibited in these ldlF cells at the restrictive temperature, consistent with a block in recycling (Hobbie *et al.,* 1994; Shima *et al.,* 1999). Unlike mammalian cells, however, yeast ε-COP/Sec28 is neither essential nor required for secretion or retrograde transport, despite being capable of binding to a KKXX motif (Duden *et al.,* 1998), so either the α and β′ subunits are the important ones of the α/β′/ε trimer (Letourneur *et al.,* 1994) or ε-COP is less important in yeast than in mammals (it is the least conserved of the COPI subunits; see Table II). Retrograde transport has been reconstituted in both yeast and mammalian systems (Spang and Schekman, 1998; Love *et al.,* 1998), so if these turn out to involve bona fide COPI vesicles, their coats could be compared for their content of ε-COP. Preliminary data also indicate that a related form of ζ, and perhaps also γ-COP, exists in a coatomer complex in mammalian cells (Whitney and Kreis, 1998), so it remains to be determined if these could also be candidates for different cargo selectors.

Importantly, the intra-Golgi transport assay (see earlier discussion) that led to the identification of COPI-coated vesicles has since been shown to function in the absence of coatomer (Elazar *et al.,* 1994) and ARF (Taylor *et al.,* 1994), conditions under which the cisternae fuse with each other, and so COPI may have an alternative or additional role in regulating tubule formation from the Golgi or preventing intracisternal fusion (see Section IV,C). Additionally, the same assay has been shown to reconstitute retrograde transport of the glycosyltransferase, which may mean that VSV-G is not transported in anterograde vesicles at all (Love *et al.,* 1998).

There is also evidence for a role of COPI in endocytosis. Not only are the ε-COP^{ts} ldlF cells defective in endocytosis (Hobbie *et al.,* 1994), but also a form of coatomer containing ε-COP but lacking stoichiometric amounts of δ- and γ-COPs has been detected on endosomal membranes, and microinjection of anti-β-COP antibodies blocks endocytic entry of viruses *in vivo* (Whitney *et al.,* 1995; Aniento *et al.,* 1996). In addition, the formation of endocytic carrier vesicles from early endosomes *in vitro* is inhibited by coatomer immunodepletion or use of ldlF ε-COP^{ts} mutant cytosol (Aniento *et al.,* 1996; Gu *et al.,* 1997). Transport to late endosomes of various fluid-phase markers, membrane-bound receptors and lysosomal proteins, and recycling of receptors from early endosomes are inhibited in ldlF cells at nonpermissive temperature (Daro *et al.,* 1997; Gu *et al.,* 1997), yet the

precise role of ε-COP in endocytosis remains to be determined. Because endocytic carrier vesicles have not been shown to be coated, COPI may instead act as a cargo sorter, regulating the segregation of cargo into endosomal tubules; as a regulator of tubule scission; or as a more structural determinant, as early endosomes in ldlF cells at restrictive temperature appear more tubular than normal and the distribution of lysosomes is also affected (Gu *et al.,* 1997; Daro *et al.,* 1997). Similar structural defects have been observed of peroxisomes in these cells (Passreiter *et al.,* 1998), but it is not clear if this implicates COPI in peroxisome biogenesis (perhaps supporting the long debated model of peroxisome biogenesis from the ER; Kunau and Erdmann, 1998) or if sorting defects lead to structural aberrations due to membrane imbalance.

Yet another role for COPI, proposed by Warren and colleagues, is the vesiculation of the rims, but not the central part, of the Golgi during mitosis (Misteli, 1996). The addition of mitotic cytosol to isolated Golgi stacks *in vitro* results in the production of COPI vesicles, which appear to carry the same cargo as those produced with interphase cytosol (Sönnichsen *et al.,* 1996), but cannot fuse due to removal of the necessary docking factor p115 by mitotic kinase-mediated phosphorylation of its proposed Golgi membrane receptor, GM130 (Nakamura *et al.,* 1997). Thus, it appears that mitotic fragmentation of the Golgi rims occurs by normal budding of COPI vesicles that are simply prevented from fusing.

4. Coatomer Interactions and Membrane Binding

There appear to be several mechanisms by which COPI coats are recruited to their donor membranes. Unlike the clathrin and COPII coats, coatomer exists in a preassembled complex in the cytosol and is recruited as such to the donor membrane when required (Hara-Kuge *et al.,* 1994). The complex is very stable, with a half-life of up to 28 h, and there is virtually no exchange of subunits (Lowe and Kreis, 1996). Only ζ-COP exists in significant quantities as a monomeric subunit in the cytosol and has been proposed to regulate coatomer binding to membranes, as anti-ζ-COP antibodies block this interaction (Kuge *et al.,* 1993). However, it is unlikely to bind directly, as bacterially expressed ζ-COP does not compete with coatomer for membrane binding.

Coatomer can be disassembled reversibly *in vitro* into subcomplexes of β′/α/ε, α/ε, β/δ, and γ/ζ (Lowe and Kreis, 1995; Pavel *et al.,* 1998) and these pairs of interactions have been confirmed by two-hybrid analysis (Faulstich *et al.,* 1996). The β′/α/ε trimer is capable of binding to KKXX fusion proteins and to Golgi membranes *in vitro* (Cosson and Letourneur, 1994; Lowe and Kreis, 1995). Furthermore, the Golgi membrane binding of the trimer can be competed specifically by KKXX fusion proteins, so the

retrograde-destined coatomer may be recruited to membranes, at least partly, by KKXX-containing cargo. γ-COP was not found as part of this membrane-binding subcomplex in high salt buffers, yet it can be cross-linked to KKXX motifs, indicating close proximity (Harter *et al.,* 1996). Experiments with aminoglycoside antibiotics, which mimic dilysine motifs and precipitate coatomer with a dose–curve similar to antigen–antibody binding, suggest that coatomer has at least two dilysine-binding sites (Hudson and Draper, 1997), so it is feasible that both α/β′/ε and γ-COP bind KKXX-containing proteins. It should be noted, however, that coatomer is not found in such subcomplexes *in vivo,* so the relevance of all these binding studies may be questionable.

Coatomer can also bind to membranes via ARF-GTP (Donaldson *et al.,* 1992a), which can be cross-linked to the β-COP subunit (Zhao *et al.,* 1997), and the isolated β/δ dimer binds membranes in an ARF-GTP-dependent fashion (Pavel *et al.,* 1998). ARF itself is recruited to the membrane on exchange of GDP for GTP by its putative Sec7 domain-containing GEF, several of which have been identified in yeast as well as in mammals (Peyroche *et al.,* 1996; Chardin *et al.,* 1996). In yeast, Gea1p and Gea2p, which exhibit BFA-sensitive activity, are required for transport between the ER and Golgi complex (Peyroche *et al.,* 1996). However, the Gea1p mammalian homolog, ARNO, is BFA insensitive (Chardin *et al.,* 1996) so may be the GEF for ARF6 (Frank *et al.,* 1998), whose recruitment is not affected by BFA (Peters *et al.,* 1995). An alternative candidate for an ARF-GEF is a 200-kDa BFA-sensitive protein (Morinaga *et al.,* 1996). Nucleotide exchange is thought to result in a conformational change that exposes the N-terminal myristic acid of ARF, allowing its insertion into the lipid bilayer (Kahn and Gilman, 1986; Nuoffer and Balch, 1994; Randazzo *et al.,* 1993). Binding of ARF to Golgi membranes *in vitro* is saturable (Helms *et al.,* 1993; Donaldson *et al.,* 1992a; Finazzi *et al.,* 1994), implying that a proteinaceous receptor for ARF also exists. The same ARF1 appears to recruit COPI to Golgi membranes (Serafini *et al.,* 1991), AP-1 to TGN membranes (Stamnes and Rothman, 1993; Traub *et al.,* 1993; Chen and Shields, 1996), AP-2 to endosomal membranes (not its normal compartment; West *et al.,* 1997), and AP-3 to the 15°C endocytic compartment that gives rise to synaptic vesicles (Faúndez *et al.,* 1998), so the ARF receptors may differ for each membrane and/or coat type. In any case, ARF has been suggested to act more as a catalyst than a stoichiometric component of the COPI coat in a BFA-resistant cell line and, as discussed in Section IV,A,2, its role may be more to do with altering lipid composition than in directly recruiting the coat (Brown *et al.,* 1993; Moss and Vaughan, 1995; Ktistakis *et al.,* 1996; but for arguments against this possibility, see Nickel and Wieland, 1997; Stamnes *et al.,* 1998). The regulation of coatomer binding is discussed further in Section V.

5. Cargo of COPI-Coated Vesicles

In the endocytic pathway, COPI has been localized to endosomal membranes and nonclathrin buds containing transferrin-HRP after a 30-min uptake, but has not yet been detected on vesicles (Whitney *et al.,* 1995), so it is not clear what their cargo is. In the biosynthetic pathway, the only well-established cargo of COPI vesicles is the KDEL receptor (Erd2p), which has been found to be concentrated in purified COPI vesicles by Western blotting (Sohn *et al.,* 1996) and electron microscopy (Sönnichsen *et al.,* 1996) and also *in vivo* by the latter technique (Orci *et al.,* 1997). The KDEL receptor recycles from its steady-state Golgi location to the ER on KDEL–ligand binding (Lewis and Pelham, 1992) and requires functional COPI for this (Lewis and Pelham, 1996), but no direct interaction has been shown.

However, the abundance of COPI and the relative efficiency of ER retention mechanisms make it unlikely that role of COPI in retrograde transport would be limited to retrieval of the low levels of escaped ER proteins. Its cargo probably also includes essential components of the transport machinery, such as v-SNAREs, folding chaperones, and cargo receptors. Although there is no direct evidence for COPI binding to SNAREs, temperature-sensitive mutations in Sec21/γ-COP and Sec27/β'-COP can be suppressed by overexpression of the ER–Golgi SNAREs Bos1, Bet1, and Sec22 (Newman *et al.,* 1990; Dascher *et al.,* 1991; Duden *et al.,* 1994), and these SNAREs have been detected in COPI-coated vesicles by electron microscopy (Bednarek *et al.,* 1995), which would be consistent with their being recycled by COPI.

Sec21/γ-COP is also necessary for recycling of the putative ERGIC-53 related chaperone Emp47p from the Golgi to the ER in yeast (Lewis and Pelham, 1996), and although no direct binding of this protein to γ-COP was shown, it does have a KXKXX motif (Schröder *et al.,* 1995) capable of such an interaction (Harter *et al.,* 1996). However, Emp47p localization is not affected in the ret1-1 (α-COP) mutant that was isolated as the first retrieval mutant of COPI. Importantly, the Sec21^{ts} mutant phenotype is very similar to that of Ufe1, which encodes the ER t-SNARE that (along with the ER proteins Sec20p and Tip20p) acts as a receptor for retrograde vesicles bearing the v-SNARE Sec22p (Lewis *et al.,* 1997). Taken together, these data may support the notion that different subunits of coatomer may regulate transport in opposite directions (Fiedler *et al.,* 1996).

Some members of the p24 family of putative cargo receptors can bind via their KKXX motifs to coatomer *in vitro* and are found in COPI-coated vesicles (Fiedler *et al.,* 1996; Stamnes *et al.,* 1995; Sohn *et al.,* 1996), supporting a role for COPI in recycling such receptors, although it seems that COPI binding to diphenylalanine motifs of the same or different p24 mole-

cules may regulate their anterograde transport too (Fiedler *et al.,* 1996; Nickel *et al.,* 1997). If the estimates that secretory cargo only represents 3% of the total membrane proteins of COPI-coated vesicles are accurate (Nickel *et al.,* 1997), this would further support the role of COPI in recycling low levels of escaped proteins rather than mediating anterograde transport. It has been shown that GTPγS, used in many experiments to prevent dissociation of the COPI coat from vesicles, can cause defects in cargo sorting (Nickel *et al.,* 1998), therefore much of the data concerning COPI cargo needs reevaluating. The inclusion of secretory VSV-G cargo in anterograde COPI vesicles has been controversial (e.g., Scales *et al.,* 1997), but pro-insulin has been detected in putative anterograde intra-Golgi COPI vesicles (Orci *et al.,* 1997).

6. A Model for COPI Action

These data can be summarized in a model for COPI action (Fig. 1). For simplicity, only the proposed role in retrograde traffic from the Golgi to the ER is shown, although COPI is also involved in other steps, as discussed earlier. The putative GTP–GDP exchange factor for ARF, ARNO in mammals or Gea1p in yeast, is somehow recruited to the membrane in a step that is inhibited by BFA. This then stimulates GTP exchange on ARF, which then binds to the membrane by a combination of insertion of its N-terminal myristate and binding to an as yet unidentified ARF receptor. This may result in the activation of phospholipase D, which can catalyze the conversion of phosphatidylcholine in the membrane to phosphatidic acid and choline, resulting in an increased negative charge, although it is not clear if this is responsible for inducing membrane curvature. ARF–GTP can also bind to β-COP, thus recruiting coatomer to the membrane, although coatomer can also bind directly to phosphatidic acid. The $\beta'/\alpha/\varepsilon$ trimer and γ-COP are also capable of interaction with KKXX motifs on membrane proteins, such as ERGIC-53 and certain members of the p24 family of candidate cargo receptors, although the latter are not always incorporated into the budded vesicle. Other membrane proteins, such as the KDEL receptor and v-SNAREs, are also likely to contact the coat or a coat–cargo adaptor, and the coat–cargo interactions are likely to be cooperative, resulting in further coatomer binding to the membrane and bud formation, although it is still unclear whether cargo stimulates coat recruitment or enters preformed buds. Coatomer most likely causes deformation of the membrane into a bud, but palmitoyl CoA is needed for periplasmic fusion, allowing the bud to pinch off into a vesicle. Although it is not clear at which stage the ARF-GAP acts, GTP hydrolysis on ARF causes it to detach from the membrane, which allows coatomer to fall off, a prerequisite for vesicle docking and fusion with the target membrane. In

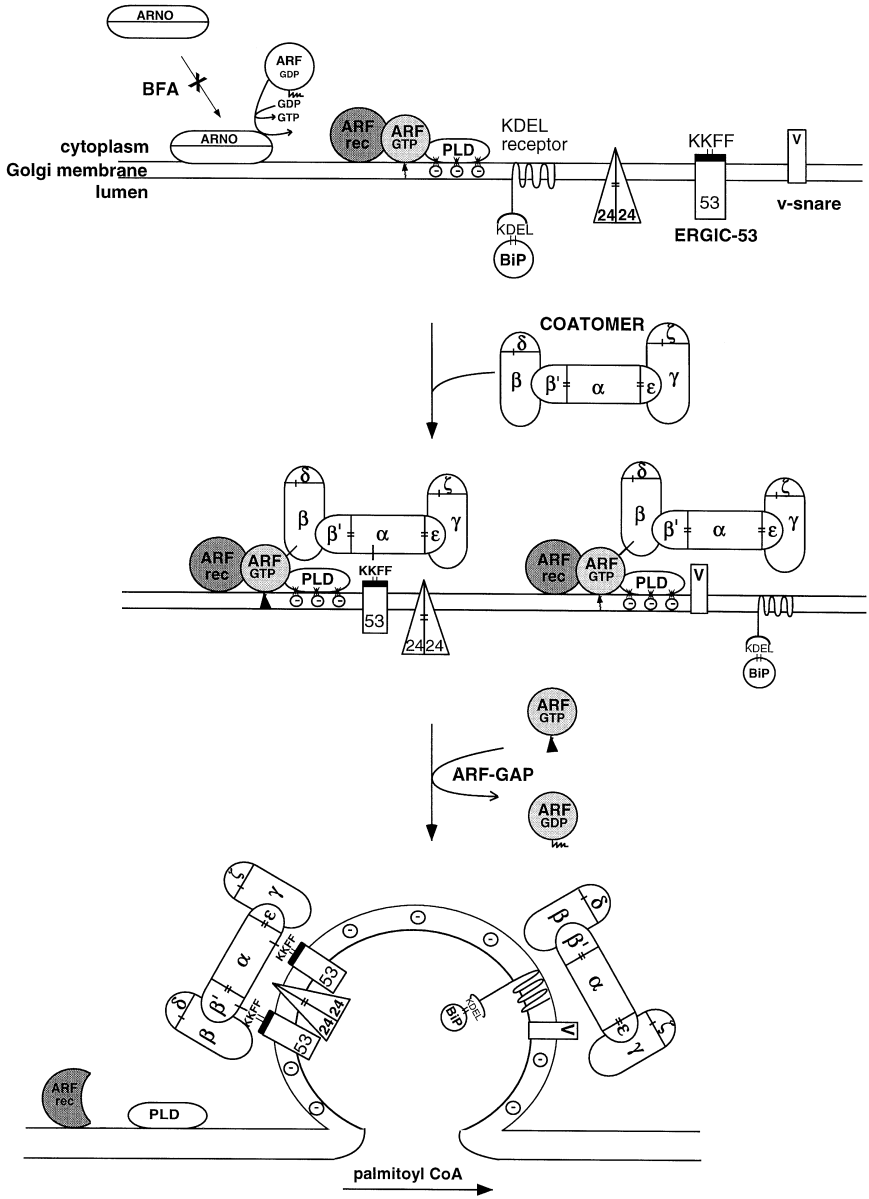

FIG. 1 COPI model of action. A current model for the sequence of events leading to coatomer recruitment to Golgi membranes, selection of cargo for recycling to the ER, and membrane deformation. See text for details. =, a stable interaction between two components of a complex; –, a more transient interaction. ARF rec, a putative membrane-bound ARF receptor; ARF-GAP, GTPase-activating protein for ARF; BiP, a soluble KDEL motif containing ER resident protein; 24, p24 family of putative coat–cargo adaptors; BFA, brefeldin A; 53, ERGIC-53 (a KKFF motif-containing lectin that faciliates transport of certain glycoproteins); v, v-SNARE (membrane protein of the docking/fusion machinery); PLD, phospholipase D; – in a circle, negatively charged phosphatidic acid formed by PLD action.

Fig. 1, ARF is depicted as activating phospholipase D rather than being a component of the coat, but there is controversy as to whether this is the case *in vivo*.

B. COPII

1. History

In contrast to COPI, the COPII proteins were not first identified as a coat, but simply by yeast genetics as proteins necessary for secretion (Novick *et al.,* 1980), later narrowed down to ER to Golgi transport (Kaiser and Schekman, 1990; Rexach and Schekman, 1991). Establishing a cell-free assay for transport of the pro-α-factor from the ER to Golgi (Baker *et al.,* 1988; Rexach and Schekman, 1991), Schekman and colleagues were able to reconstitute vesicle formation from urea-washed ER membranes using three soluble protein complexes: Sec23p/Sec24p complex, Sec13p/Sec31p complex, and the small GTPase Sar1p (Salama *et al.,* 1993). They subsequently showed that these formed a 10-nm fuzzy coat on 60- to 65-nm vesicles (Barlowe *et al.,* 1994), distinct from COPI-coated 70- to 75-nm vesicles, which exclude pro-α-factor (Bednarek *et al.,* 1995). These COPII-coated vesicles were functional in that they excluded resident ER proteins such as Kar2p (BiP) and Sec12p, and once uncoated they could fuse with an acceptor Golgi fraction if cytosol (including functional Ypt1p) was added. However, they were not identical to COPII vesicles produced using crude cytosol (Lian and Ferro Novick, 1993) in that they lacked Ypt1p (since none was added) and contained larger amounts of the SNARE Bet1p (Salama *et al.,* 1993; Barlowe *et al.,* 1994). Ypt1p normally regulates the interaction of Bet1p with the v-SNARE Bos1p (Stone *et al.,* 1997), so it is not completely clear whether the vesicles produced with purified COPII components undergo nonphysiological fusion with the Golgi due to altered SNARE interactions. Alternative fusion partners could be other COPII vesicles (supporting the maturation model) or the intermediate compartment, but unlike in mammalian cells, there is no strong evidence for the existence of this compartment in yeast (Pelham, 1995). A dozen or so proteins that are sufficiently concentrated in COPII vesicles to be detected by silver staining and may be components of the vesicle budding or targeting machinery are still awaiting identification.

2. Components of the COPII coat

All the components of the yeast COPII coat identified so far have now been cloned, as well as some of their homologs in other species (Table

III). Unlike COPI, the COPII coat is not incorporated *en bloc* to the membrane, but sequentially in the order Sar1p-GTP, Sec23p/Sec24p complex, and Sec13p/Sec31p complex (Matsuoka *et al.,* 1998b; Aridor *et al.,* 1998). Sec16p appears to organize coat assembly without itself being a typical coat protein (Shaywitz *et al.,* 1997).

Sar1p is a small 21K GTPase that was identified as a high-copy suppressor of the Sec12 mutation (Nakano and Muramatsu, 1989). ER resident Sec12p recruits Sar1p to the ER membrane and acts as its specific activating guanine nucleotide exchange factor (GEF; d'Enfert *et al.,* 1991; Barlowe and Schekman, 1993), without forming part of the COPII coat. Sar1p-GTP is responsible for recruiting the Sec23p/Sec24p complex to the membrane, perhaps via the GTPase-activating domain of Sec23p, which is specific for promoting the hydrolysis of GTP on Sar1p (Yoshihisa *et al.,* 1993). GTP binding to Sar1p, but not hydrolysis by Sec23p, is necessary for budding, but hydrolysis must occur to allow uncoating and subsequent fusion (Barlowe *et al.,* 1994). Sar1p homologs have also been identified in other species, including *Schizosaccharomyces pombe, Arabidopsis thaliana* (d'Enfert *et al.,* 1992), and two in mammalian cells, which have been shown to activate budding from ER but not Golgi membranes (Sar1a and Sar1b; Kuge *et al.,* 1994). Yeast Sar1p is also 34% homologous to yeast ARF, its closest relative in the small GTPase family (Nakano and Muramatsu, 1989), supporting its role as a coat protein recruiter. This is the only sequence homology between COPII and COPI other than the WD-40 motifs (see later).

Sec23p is an 85K GAP (GTPase activating protein), unrelated to other GAPs, that is recruited by and acts specifically on Sar1p (Yoshihisa *et al.,* 1993). It is only active for budding as a 400K heterodimer with the 105K Sec24p (Hicke *et al.,* 1992), which has no effect on the GAP activity of Sec23p. Because GTP hydrolysis by Sec23p is not necessary for budding, other roles of Sec23p/Sec24p may include interactions with secretory cargo or their receptors and v-SNAREs, as well as binding to Sec16p (Espenshade *et al.,* 1995) and recruiting the Sec13p/Sec31p complex to the nascent bud. Indeed the Sec23p/24p and Sar1p subunits of the COPII coat have been shown to be in a complex in the yeast ER membrane with the v-SNAREs Sec22p and Bet1p; the putative cargo receptor Emp24p; the amino acid permeases Hip1p and Gap1p; and luminal pro-α-factor cargo, which is cross-linkable to two putative transmembrane cargo adaptors of 19 and 27K (Kuehn *et al.,* 1998). Moreover, a direct interaction between the Sec23p/Sec24p complex and the SNAREs Bos1p and Bet1p has been detected *in vitro,* dependent on the presence of Sar1p-GTP (Springer and Schekman, 1998), and Bos1p and Sec22p incorporated into liposomes could be concentrated into vesicles by COPII components (Matsuoka *et al.,* 1998a). Similarly, the transmembrane cargo VSV-G can be found in a prebudding complex with mammalian Sar1-GTP/Sec23 (Aridor *et al.,* 1998) and is

TABLE III

COPII Subunits in Yeast and Their Mammalian Homologs

COPII subunit	Size (kDa)	Reference	Mammalian homolog	Source	Reference	% identity
Sec13p	34	Pryer *et al.* (1993)	Sec13	Human	Swaroop *et al.* (1994)	53
Sec31p	150	Salama *et al.* (1997)				
Sec23p	85	Hicke and Schekman (1989)	hSec23a	Human B cell	Paccaud *et al.* (1996)	48
			hSec23b	Human B cell	Paccaud *et al.* (1996)	48
Sec24p	105	Unpublished (Schekman laboratory)	hSec24a	Human	Pagano *et al.* (1999)	28
			hSec24b	Human	Pagano *et al.* (1999)	26
			hSec24c	Human	Pagano *et al.* (1999)	24
			hSec24d	Human	Pagano *et al.* (1999)	25
Sar1p	21	Nakano and Muramatsu (1989)	Sar1a	CHO cells	Kuge *et al.* (1994)	61
			Sar1b	CHO cells	Kuge *et al.* (1994)	61

thought to be concentrated in these vesicles compared to the donor ER membrane (Balch *et al.,* 1994). So, it seems that the COPII coat really can interact with cargo, at least indirectly through adaptors, if not directly in the case of some transmembrane cargoes.

Two mammalian homologs of Sec23 have been found, Sec23a and Sec23b, but only Sec23a can complement Sec23-deficient yeast (Paccaud *et al.,* 1996) and this localizes to the ER transitional zone and COPII vesicles (Orci *et al.,* 1991a). The localization and function of Sec23b and its yeast homologs are not yet known. There are two weakly related Sec24 genes in yeast, one of which, Lst1p, interacts with all the COPII genes and so far appears to specifically package Pma1p into ER-derived vesicles (Roberg *et al.,* 1999). There are at least four Sec24 genes in human (hSec24a, b, c, and d; Pagano *et al.,* 1999). Importantly, coimmunoprecipitation studies show that hSec23a forms mutually exclusive complexes with hSec24b and hSec24c, and these may also select different cargo at the ER, as all three proteins localize to the ER/intermediate compartment (Pagano *et al.,* 1999).

Sec13p is a 34K protein (Pryer *et al.,* 1993) that exists as a 700K heterodimer (Salama *et al.,* 1993) with the 150K Sec31p protein (Salama *et al.,* 1997). Sec13p/Sec31p is recruited to the membrane by the Sec23p/Sec24p complex and appears to initiate the final steps of budding, perhaps by inducing membrane deformation and pinching off (Kuehn *et al.,* 1998; Aridor *et al.,* 1998). Serine phosphorylation of the Sec13p/31p complex may help regulate budding, as the dephosphorylated complex inhibits budding *in vitro* (Salama *et al.,* 1997). Whereas the mammalian homolog of Sec13 (Swaroop *et al.,* 1994) does not fully complement a yeast Sec13 defect (Shaywitz *et al.,* 1995), it does compete with it for budding (Tang *et al.,* 1997), so is likely to be part of a functional mammalian COPII coat. Consistent with this, mammalian Sec13 is more punctate than the ER-localized Sec23 and localizes mainly to the intermediate compartment and vesicles (Tang *et al.,* 1997), supporting the notion that COPII in mammalian cells acts between the ER and the intermediate compartment rather than the ER and Golgi (Aridor *et al.,* 1995; Scales *et al.,* 1997). The only other known homolog of Sec13p is the yeast Seh1p protein, which does not affect ER to Golgi transport of the COPII cargo carboxypeptidase Y and appears to be part of the nuclear pore complex, along with some of Sec13p itself, so most likely has a different function (Siniossoglou *et al.,* 1996). No homologs have yet been reported for Sec31p, but both this and Sec13 contain seven and six WD-40 repeats, respectively, akin to those in COPI's α- and β'-COPs and the G$\beta\gamma$ subunit of heterotrimeric G-proteins, but whether these simply allow dimerization or have a regulatory function is still unclear (Neer *et al.,* 1994). Sec13p has been implicated in the nitrogen-regulated TGN–plasma membrane transport of Gap1p, as well as in ER–Golgi transport, so there may be another COPII-related coat to be discovered (Roberg *et al.,* 1997b).

Other genes affecting this pathway (Lst4, Lst7, and Lst8) appear not to be homologous to other COPII components, but whether they are part of this potentially new coat remains to be determined (Roberg *et al.,* 1997a).

Sec16 mutants have the same lack of budding phenotype as Sec12, Sec13, and Sec23 and show synthetic lethal interactions with these genes (Kaiser and Schekman, 1990), yet this gene product was not required in Schekman's budding assay, as it remains bound to ER membranes after the urea wash that removes the COPII coat components. The 240K Sec16p binds via distinct domains to Sec23p, Sec24p, and Sec31p, but appears not to cycle on and off the membrane (Espenshade *et al.,* 1995; Gimeno *et al.,* 1995, 1996; Shaywitz *et al.,* 1997), so although it is a peripheral component of COPII vesicles, it does not behave as a typical coat protein. In addition, Sec16 mutants are more defective in packaging v-SNARE and membrane cargo than luminal cargo *in vitro* (Campbell and Schekman, 1997), so it may act as a cargo sorter or more likely as a scaffolding protein for COPII recruitment. If Sec16p does indeed organize coat assembly, as proposed from its interactions with the COPII subunits (Shaywitz *et al.,* 1997), it may influence the surface area to volume ratio of the vesicles, thus explaining the observed change in the proportion of membrane-bound to soluble cargo. Indeed, COPII-coated vesicles formed in the absence of Sec16p and other membrane proteins are deformed and heterogeneous in size (Matsuoka *et al.,* 1998b). Interestingly, Sed4 was cloned as a high-copy suppressor of Sec16 and is homologous to Sec12, the GEF for Sar1p. Like Sec12p, Sed4p is localized to the ER (it is a rare example of a membrane protein bearing a C-terminal HDEL signal), does not enter COPII vesicles, and interacts genetically with Sar1p. However, it does not stimulate nucleotide exchange on Sar1p, so its function remains a mystery and it is not yet clear why two such proteins should be required (Gimeno *et al.,* 1995).

3. Cargo of COPII-Coated Vesicles

Although COPII-coated vesicles can be formed *in vitro* in the absence of other proteins than Sarlp-GTP and the Sec23p/24p complex (Matsuoka *et al.,* 1998b), this is unlikely to occur *in vivo.* The contents of COPII vesicles can be divided into three categories: secretory cargo being transported from the ER to the Golgi, components of the folding and sorting machinery, and components of the targeting and docking machinery. Of these, only the short-lived secretory cargo is likely to be depleted from cells by cycloheximide treatment, thus that at least some COPII-coated vesicles can form under such conditions does not imply that no proteins are included at all (Aridor *et al.,* 1999).

Secretory cargo shown to be transported in COPII but not COPI vesicles in yeast includes soluble pro-α-factor (Bednarek et al., 1995), carboxypepti-

dase Y, and periplasmic invertase. Nonsoluble secretory cargo includes GPI-anchored proteins such as Gas1p, whose entry is dependent on the GPI moiety (Doering and Schekman, 1996), and transmembrane proteins such as alkaline phosphatase, the plasma membrane ATPase, Pma1p, and the amino acid permeases Hip1p and Gas1p, whose entries are dependent on the ER resident putative amino acid permease sorter, Shr3p (Kuehn *et al.,* 1996). In mammalian cells, where it seems more likely that COPII only functions between the ER and the intermediate compartment, COPII cargo has been shown to include the transmembrane VSV-G protein (Aridor *et al.,* 1995). For some transmembrane cargo, including VSV-G, a diacidic (D/E-X-D/E) motif downstream of the tyrosine-containing basolateral motif has been shown to facilitate export from the ER (Nishimura and Balch, 1997), although it appears that this motif is not responsible for the observed interaction of VSV-G with Sec23.

In the folding/sorting machinery category of COPII vesicle proteins in yeast there is the putative cargo receptor Emp24p, which affects the transport of periplasmic invertase and Gas1p, but not of pro-α-factor, carboxypeptidase Y, or acid or alkaline phosphatases (Schimmöller *et al.,* 1995). Emp24p must form a (probably stoichiometric) complex with one of its homologs, Erv25p, in order for both to be incorporated into COPII vesicles to mediate these sorting events (Belden and Barlowe, 1996). Erv25p has a KXKXX motif in its cytoplasmic domain, which may provide a means of recycling the complex in COPI vesicles, but this has yet to be tested. Only one p24 family member has been associated with COPII vesicles in mammals, the rest being in COPI-coated vesicles (Sohn *et al.,* 1997). Mammalian ERGIC-53/p58 has been shown to be concentrated into COPII-coated vesicles (Rowe *et al.,* 1996), and its cytoplasmic diphenylalanine motif can interact *in vitro* with Sec23/Sec24 (Kappeler *et al.,* 1997). It may function as a mannose-specific chaperone or cargo receptor (Arar *et al.,* 1995) and is likely to be recycled to the ER in COPI-coated vesicles.

The docking machinery of COPII vesicles includes the v-SNAREs Bos1p, Sec22p (Sly2p), and probably also Bet1p (Sly12p), although there has been controversy as to inclusion of the latter (Rexach *et al.,* 1994; Barlowe *et al.,* 1994; Lian and Ferro Novick, 1993), and data suggest it may function more as a SNAP25-like t-SNARE than a v-SNARE (Stone *et al.,* 1997). The rab-related protein Ypt1p is recruited to the vesicle membrane and is necessary for the activation of the v-SNARE Bos1p on COPII vesicles (Lian *et al.,* 1994), so the as yet undiscovered Ypt1p effector and putative receptor are also likely to be COPII vesicle-associated proteins.

4. A Model for COPII Action

All these data can be summarized in the following model for COPII budding from the ER (Fig. 2; see also Bednarek *et al.,* 1996; Kuehn and Schekman,

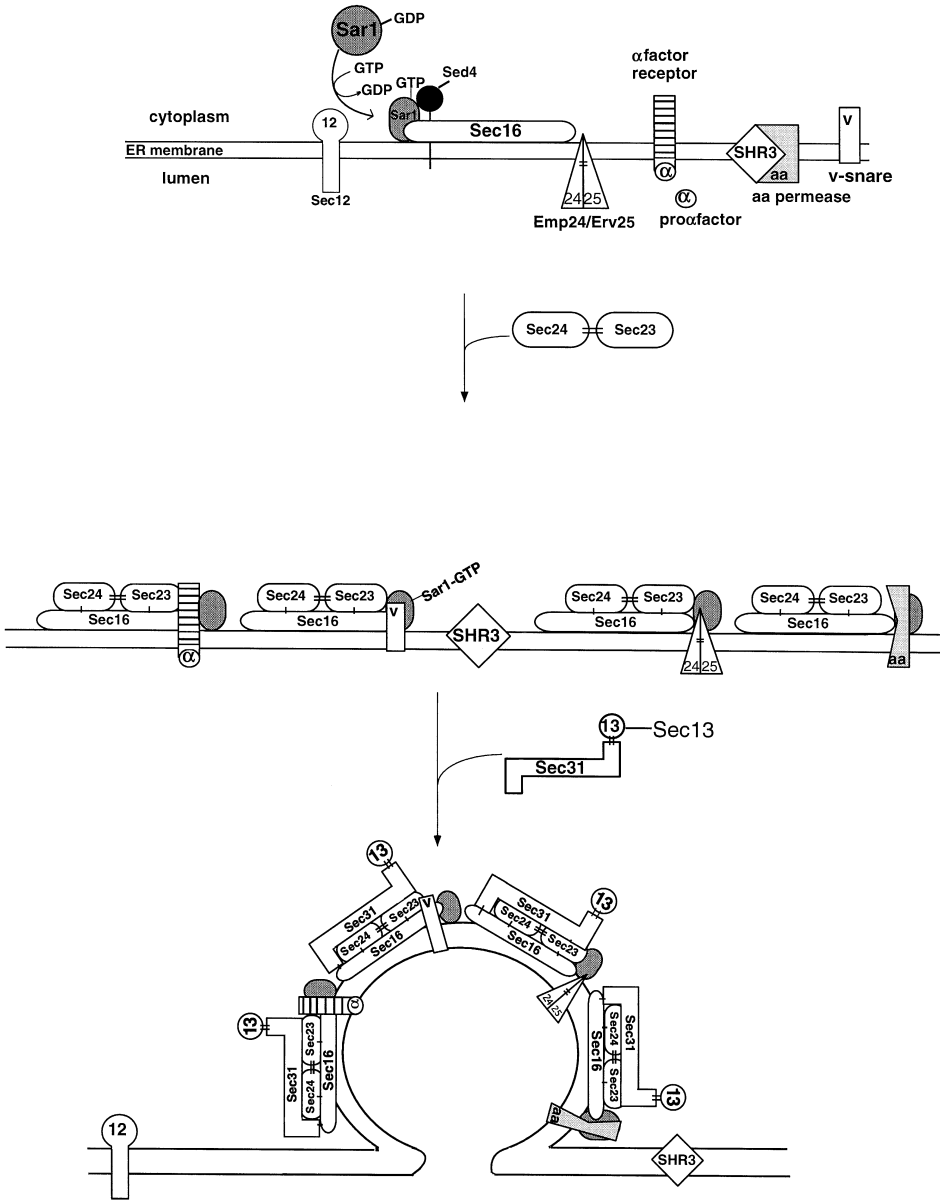

FIG. 2 COPII model of action. The sequence of events that is currently thought to lead to coat recruitment to the ER membrane, cargo selection, and membrane deformation by COPII components. See text for details. =, a stable interaction between two components of a complex; –, a more transient interaction. 12, Sec12p; 24, 25, Emp24p/Erv25p oligomer (an example of the p24 family of putative coat–cargo adaptors); α, pro- α -factor (an example of soluble secretory cargo); aa, amino acid permease (whose transport is facilitated by Shr3p); v, v-SNARE (membrane protein of the docking/fusion machinery); 13, Sec13p. Other proteins are as labeled.

1997). Sec12p in the ER membrane recruits Sar1p by exchanging its GDP for GTP. Sar1p may be stabilized on the membrane by interactions with Sec16p and Sed4p, although the Sed4p interaction is transient, as only Sec16p is subsequently incorporated into the vesicle. The Sec23p/Sec24p complex then binds to Sar1p-GTP and the membrane-associated Sec16p, which probably acts as a scaffold facilitating COPII recruitment. The Sec23p/Sec24p–Sar1p-GTP complex also contacts integral membrane proteins that are to be included into the vesicle, such as cargo (e.g., amino acid permeases, which require Shr3p for entry but leave Shr3p behind, and VSV-G), cargo receptors (e.g., Emp24p), and SNAREs (e.g., Sec22p, Bos1p, and Bet1p). Binding of the Sec13p/Sec31p complex to Sec23p/Sec24p, and also of Sec31p to Sec16p, ultimately results in vesicle budding, probably by deforming the membrane. No analog of the palmitoyl Co-A needed for the pinching off of COPI vesicles appears to be necessary, although it could be a component of urea-washed ER membranes. GTP binding by Sar1p, but not its hydrolysis, is necessary for budding and it is not yet clear at which point GTP hydrolysis is catalyzed by the GAP activity of Sec23p to stimulate uncoating. Uncoating allows the v-SNAREs Sec22p, Bos1p, and Bet1p to bind to their cognate t-SNARE Sed5p/Syntaxin5 on the acceptor membrane to permit docking and subsequent fusion with the Golgi or intermediate compartment. Ypt1p/rab1 is necessary to somehow activate the v-SNAREs to permit this docking. Still awaiting discovery are what regulates Sec12p to define the location and timing of budding; the role of the Sec12p homolog Sed4p; what restrains the GAP activity of Sec23p until the vesicle has budded; how the putative cargo receptors Emp24p and Shr3p sort cargo into the vesicle; and precisely how the v-SNAREs are activated by Ypt1p on inclusion into the vesicle via interaction with the coat. Despite all these unknowns, there is a clear consensus for COPII action in the concentration and transport of selected cargo from the ER to the intermediate compartment or Golgi in coated vesicles (see later), although there are hints that COPII may play other roles too.

IV. Models for Membrane Traffic in Eukaryotic Cells

A. The Vesicle Shuttle Model

The vesicle shuttle model, or stable compartment model, views intracellular compartments as being relatively stable structures, which transfer material by means of transport vesicles, and has enjoyed the most support in recent years. Because the general mechanism of action of the clathrin, COPI, COPII, and AP-3 coats described earlier is very similar, we will discuss

their potential roles in the budding and targeting of transport vesicles together. These roles include selection of cargo, deformation of the membrane, interaction with the targeting machinery, and regulation of docking.

1. Selection of Cargo

Exactly how coat proteins are recruited to specific sites on the donor membrane to initiate budding is unclear. Because the ultimate goal of the coated vesicle is to transport cargo from one compartment to another, the idea that the cargo itself could stimulate coated vesicle formation is logical. However, such stimulation must be regulated because the steady-state distribution of the coat proteins does not parallel the distribution of their cargo. For example, ~70% of the transferrin receptor is found in early endosomes, whereas the AP-2 adaptor that transports it there predominates at the plasma membrane. Similarly, COPI is localized mostly to the *cis*-Golgi/intermediate compartment, whereas most of its KKXX-containing cargo resides in the ER.

a. Selection of Cargo by Adaptor Complexes The classic example of cargo stimulating coated vesicle formation is in the endocytic pathway, where certain endocytosed proteins were shown to be concentrated in clathrin-coated pits, with the first completely resolved interaction being between the clathrin adaptor AP-2 and the tyrosine-containing cytoplasmic tails of LDL receptors (Anderson *et al.,* 1977; Pearse, 1981; Trowbridge *et al.,* 1993). All of the clathrin-coated endocytic vesicles visualized so far by electron microscopy certainly appear to be completely filled with cargo (e.g., Geuze *et al.,* 1984). However, it has since been questioned whether the adaptor–cargo interaction stimulates clathrin recruitment or simply sorts the cargo into a preformed coated bud (Fire *et al.,* 1995; Santini and Keen, 1996). Transferrin binding to the transferrin receptor is not required for receptor internalization (Watts, 1985), but overexpression of the transferrin receptor itself certainly increases clathrin polymerization at the plasma membrane, although there is controversy as to whether this increases the number of coated pits or only flat lattices (Iacopetta *et al.,* 1988; Miller *et al.,* 1991). Similarly contradictory results have been obtained for the involvement of AP-2 in (the ligand-dependent) EGF receptor internalization: AP-2 complexes can be coimmunoprecipitated in stoichiometric amounts with the receptor (Sorkin *et al.,* 1995), but inhibition of the AP-2 interaction with the receptor has no effect on its internalization (Nesterov *et al.,* 1995). This discrepancy may be explained by the ability of cells to switch to alternative clathrin-independent means of endocytosis when clathrin-mediated endocytosis is impaired (Sandvig and van Deurs, 1994). The strongest argument against cargo being absolutely necessary for cla-

thrin recruitment is the demonstration that clathrin alone can generate vesicles from protein-free liposomes (Takei *et al.,* 1998). However, these conditions were far from physiological and *in vivo* membrane proteins are most likely to regulate coat recruitment. The authors therefore concluded that membrane proteins are necessary for the temporal and spatial regulation of vesicle budding, whereas clathrin provides the deformative force.

The case for AP-1 adaptors being recruited by cargo proteins is also convincing. When the major cargoes of TGN-derived clathrin vesicles, the two mannose-6-phosphate receptors (which themselves function as cargo receptors for soluble lysosomal enzymes bearing a mannose-6-phosphate signal) were knocked out in mice, the result was decreased recruitment of AP-1 adaptors and clathrin to TGN membranes (Le Borgne *et al.,* 1996). Moreover, the reexpressed mannose-6-phosphate receptor recruited AP-1 and increased the number of clathrin-coated vesicles as a function of its concentration, provided the cytoplasmic tail containing the endosomal sorting determinants, the dileucine motif, and a casein kinase II phosphorylation site was intact (Le Borgne and Hoflack, 1997), strongly implicating these cargo receptors as stimulators of TGN–clathrin vesicle formation. The functional analog of mannose-6-phosphate receptors in yeast is Vps10p, a transmembrane receptor that sorts carboxypeptidase Y by directly binding its vacuolar sorting signal (QRPL), as well as protease A via an unrelated signal, facilitating their transport from the TGN to the vacuole (Marcusson *et al.,* 1994; Cooper and Stevens, 1996). The coat mediating this step is likely to be clathrin, as carboxypeptidase Y is missorted in a clathrin heavy chain mutant, and the FYVF sequence in Vps10p is similar to the tyrosine/bulky hydrophobic motifs necessary for clathrin adaptor binding (Horazdovsky *et al.,* 1995).

Extensive studies have shown that AP-1 and AP-2 complexes can interact with tyrosine and dileucine motifs (Marks *et al.,* 1997; see also Heilker *et al.,* 1996; Honing *et al.,* 1996; Boll *et al.,* 1995; Ohno *et al.,* 1995). Furthermore, the binding of AP-2 to tyrosine-based motifs was shown to be strengthened on clathrin coat formation, suggesting that cargo recognition and coat assembly are indeed coupled (Rapoport *et al.,* 1997). The newly discovered yeast AP-3 complex has similarly been shown to mediate the transport of only selected cargo, such as alkaline phosphatase and the vacuolar t-SNARE Vam3p, from the late Golgi to the vacuole, and the dileucine motif of alkaline phosphatase is necessary for sorting into this novel pathway (Cowles *et al.,* 1997a,b; Stepp *et al.,* 1997). In addition, mammalian AP-3 recruitment to membranes of the perinuclear region has been shown to be increased on the overexpression of reporters bearing the cytoplasmic tails of LIMP-II and LAMP-I, dependent on the presence of their dileucine and tyrosine motifs, respectively (Le Borgne *et al.,* 1998).

It is thus apparent that the adaptor complexes bind to cytoplasmic dileucine and tyrosine sorting signals important for endocytosis, lysosomal targeting, and basolateral targeting from the TGN (Letourneur and Klausner, 1992; Mellman, 1996; Heilker *et al.,* 1996; Kirchhausen *et al.,* 1997). Two-hybrid system data have pinpointed the tyrosine motif interaction to the μ subunits of all three adaptor complexes (AP-1 and AP-2: Ohno *et al.,* 1995; AP-3: Dell'Angelica *et al.,* 1997). Because the amino acids surrounding the critical tyrosine are important in defining the binding specificity of $\mu 1$ and $\mu 2$, recognition by these adaptor complexes may well be a strong determinant of sorting from the TGN to lysosomes or internalization at the plasma membrane, respectively (Marks *et al.,* 1997). The μ subunits bind tightly to the β subunits (Page and Robinson, 1995; Seaman et al., 1996), which in turn recruit clathrin in the case of AP-1 and AP-2 (Gallusser and Kirchhausen, 1993; Shih *et al.,* 1995), if not also AP-3 (Dell'Angelica *et al.,* 1998). Posttranslational modifications, such as phosphorylation, may also determine the affinity of interaction between the targeting signals and the adaptors (Mauxion *et al.,* 1996; Boll *et al.,* 1996), and combinations of different motifs may also strengthen the interaction, as in the case of mannose-6-phosphate receptors, which contain both tyrosine-based and dileucine-based motifs (Kornfeld, 1992). Due to the low affinity of both tyrosine and dileucine motifs for the adaptors ($K_d \sim 10\ \mu M$), however, it seems unlikely that these by themselves account for the specificity of adaptor recruitment. Factors other than cargo may therefore also be involved in adaptor recruitment, as already discussed in Section II,A,3.

b. Selection of Cargo by COP Coats By analogy, one would expect similar interactions between nonclathrin COP coats and cargo proteins to occur in the secretory pathway. However, a direct interaction between secretory cargo and coat proteins (or an indirect one via coat–cargo adaptors) has yet to be shown, although there are indications that this may be the case (see later). An argument against this idea is that cycloheximide treatment does not completely prevent COPII budding from the ER (Kaiser and Schekman, 1990; Aridor *et al.,* 1999) nor COPI from the Golgi (Orci *et al.,* 1986), although such treatment was unlikely to have completely depleted the secretory pathway of secretory cargo. Moreover, as for clathrin, vesicles have been formed simply by adding purified COPII components or coatomer and ARF-GTP to artificial pure acidic-lipid membranes (Matsuoka *et al.,* 1998b; Spang *et al.,* 1998), so cargo appears not to be obligatory, at least under nonphysiological conditions. However, more physiological lipids require p24 proteins to recruit COPI (Bremser *et al.,* 1999), and secretory cargo is concentrated selectively into ER-derived (but probably not intra-Golgi)-coated vesicles (Mizuno and Singer, 1993; Rexach *et al.,* 1994; Balch *et al.,* 1994; Bannykh *et al.,* 1996), implying that it is sorted actively and

not simply incorporated by bulk flow. Furthermore, when ER-Golgi v-SNAREs and ER resident proteins are incorporated into the liposomes mentioned previously, COPII components bind selectively to the v-SNAREs and concentrate them into vesicles, suggesting that COPII proteins are sufficient for v-SNARE selection (Matsuoka *et al.,* 1998a). In addition, retention of the temperature-sensitive secretory cargo, VSV-G, in the ER of infected cells (where the VSV virus prevents host protein cargo synthesis) leads to a decrease in ER-derived vesicle formation, despite its continued binding to Sec23 in a prebudding complex (Aridor *et al.,* 1999). The KDEL receptor has similarly been shown to stimulate COPI recruitment on ligand binding, reportedly through the regulation of ARF-GTPase-activating protein activity (Aoe *et al.,* 1997, 1998). Further evidence against the bulk flow hypothesis for ER export includes the identification of specialized ER exit sites (export complexes; Bannykh *et al.,* 1996), an ER export signal in certain cargo (Nishimura and Balch, 1997), and several candidate sorting proteins, discussed later.

Certain COPII subunits have been found in a complex with integral membrane and soluble cargo molecules, both in the ER membrane and in COPII vesicles (Kuehn *et al.,* 1998; Aridor *et al.,* 1998). Whereas these interactions may be direct in the case of v-SNAREs and other transmembrane proteins (Springer and Schekman, 1998; Matsuoka *et al.,* 1998a), candidate cargo receptors or sorting adaptors are being discovered for both integral membrane and soluble cargoes. One such candidate is the p24 family (Kuehn *et al.,* 1998; Stamnes *et al.,* 1995; Blum *et al.,* 1996), whose so far discovered members in yeast are associated with and facilitate the budding of COPII-coated vesicles: Emp24p (Schimmöller *et al.,* 1995) and Erv25p (Belden and Barlowe, 1996) form a heterooligomer and only a subset of cargo is delayed significantly in transport when they are mutated or deleted, suggesting that they may act as cargo facilitators. A genetic link to the COPII coat has been shown for Emp24/Bst2 and two other Bst proteins in that mutations in these can suppress a defect in Sec13 (Elrod Erickson and Kaiser, 1996). It is not completely clear, however, whether these negative regulators of COPII budding act by allowing sorting of cargo or by creating a structural subdomain of the ER to permit budding only at specific sites. Emp24p/Bst2p can be found in the complex mentioned previously in the ER membrane and in COPII vesicles with Sec23p/Sec24p and Sar1p (Kuehn *et al.,* 1998), perhaps supporting the former possibility.

In mammalian cells, only one p24 family member has been associated so far with COPII-coated vesicles (Sohn *et al.,* 1997); the rest are mostly associated so far with COPI-coated vesicles (Sohn *et al.,* 1996), and at least some of them interact with coatomer via their phenylalanine and/or dilysine motifs *in vitro* (Fiedler *et al.,* 1996; Stamnes *et al.,* 1995), although they are also capable of binding to Sec23 *in vitro* (Dominguez *et al.,* 1998). Cross-

linking experiments, sedimentation analysis, and redistribution of overexpressed p24 family members on mutation of other family members indicate that like Emp24p/Erv25p, members of the mammalian p24 family can also form heterooligomeric complexes (Sohn *et al.,* 1997; Dominguez *et al.,* 1998). However, no interactions with cargo have been found so far nor has it been shown whether the p24 proteins actively recruit coatomer to membranes *in vivo,* although they can *in vitro* (Bremser *et al.,* 1999). The fact that yeast Emp24p can bind to coatomer as well as COPII via its presumed anterograde diphenylalanine signal *in vitro* (Fiedler *et al.,* 1996; Dominguez *et al.,* 1998), despite only being found in COPII vesicles *in vivo* for anterograde transport (Schimmöller *et al.,* 1995), illustrates that such *in vitro* studies may not always reveal physiologically relevant interactions, as one would predict any Emp24p recycling in COPI-coated vesicles to be mediated through the dilysine motif of its binding partner, Erv25p. In addition, one member of this family, BHK cell p23, appears neither to be incorporated into COPI-coated vesicles nor to bind to COPI components, remaining instead in the intermediate compartment while still being important for VSV-G export from there (Rojo *et al.,* 1997). Its abundance led the authors to propose a strutural role for p23, although clearly other functions must be evoked in order to explain the variation in the luminal domains of the different members of this growing family, which would be more consistent with their proposed roles as receptors for different cargoes. A newly characterized yeast COPII vesicle protein that may function in a similar way to Emp24p is Erv14p, which so far appears to be specifically required for transport of the axial bud site selection protein, Ax12p (Powers and Barlowe, 1998).

Another candidate cargo receptor is Shr3p, necessary for the packaging of integral membrane amino acid permeases (but not other integral membrane proteins) into COPII vesicles in yeast (Kuehn *et al.,* 1996), but so far it is unclear if this protein binds directly to either the permeases or to COPII, as it does not enter COPII vesicles, nor is it detected in the Emp24p-containing ER membrane complex with COPII proteins (Kuehn *et al.,* 1998). Because the amino acid permeases themselves (at least Hip1p and Gap1p) are part of this COPII complex, it is most likely that Shr3p acts before their recognition by COPII rather than as a coat–cargo adaptor, its function perhaps being to shunt them toward a preformed coated bud site, to initiate coat recruitment without subsequent entry into the bud, or to simply act as a permease-specific quality control chaperone. The same group has also identified two candidate transmembrane sorting receptors of 19 and 27K that can be cross-linked to soluble α-factor cargo (Kuehn *et al.,* 1998).

A newly emerged candidate for a transmembrane cargo receptor is BAP31, which binds specifically to a small pool of cellubrevin and only

partially colocalizes with it in the ER when cellubrevin is overexpressed, leading the authors to propose a role for BAP31 in the sorting of newly synthesized cellubrevin (Annaert *et al.,* 1997). However, as for Shr3p, a role as a quality control chaperone or a masking factor to prevent newly synthesized cellubrevin-containing vesicles from fusing inappropriately cannot yet be excluded. No interactions with coat proteins have yet been investigated, although it is interesting to note that the KKXX motif of BAP31 appears not to affect its ER localization. Significantly, BAP31 appears to belong to a conserved family of proteins whose members have been shown previously to bind to immunoglobulins (e.g., Kim *et al.,* 1994), which may be more consistent with a sorting or chaperone function than a masking one.

Another candidate receptor is the Golgi-localized Rer1p, which facilitates the retrieval of escaped Sec12p, Sec63p, and Sec71p to the ER in yeast (Sato *et al.,* 1997; Boehm *et al.,* 1997). It appears to be the transmembrane domain rather than the cytoplasmic domain of Sec12p that is recognized by Rer1p, and COPI is implicated in their retrograde transport, as the ret1-1 mutation in α-COP affects their ER localization.

The need for a cargo receptor to link soluble luminal cargo to the cytosolic coat proteins is obvious, but the only advantages to having a coat cargo adaptor for transmembrane cargo would be to increase the fidelity of sorting or if the adaptors could recognize a wide variety of cargo, such as Shr3p appears to help package all 17 amino acid permeases. In this respect, one may consider that the quality control chaperones could act as wide-range cargo receptors, as although they mainly ensure that only properly folded proteins leave the ER, many of them have been shown to cycle between the ER and CGN, perhaps in the process of escorting cargo (Hammond and Helenius, 1995). Examples include BiP/Kar2p (Munro and Pelham, 1986), Hsp47, cyclophilin B (Smith *et al.,* 1995), and the more specific Vma21p, one of the proteins that controls the assembly of the vacuolar ATPase (Hill and Stevens, 1994).

Another candidate for a general cargo receptor for ER–Golgi traffic via COPII and COPI vesicles is the mannose-binding lectin ERGIC-53/p58 and its homologs (Arar *et al.,* 1995; Itin *et al.,* 1996; Fiedler and Simons, 1994). ERGIC-53 is concentrated into COPII-coated vesicles (Rowe *et al.,* 1996), perhaps through interaction of its cytoplasmic diphenylalanine motif with Sec23p/Sec24p (Kappeler et al., 1997); may bind to core-glycosylated cargo (Itin *et al.,* 1996; Schindler *et al.,* 1993); has a KKFF motif with the potential to bind COPI (Tisdale *et al.,* 1997); is found in COPI vesicles (Sönnichsen *et al.,* 1996); and cycles continuously between the ER and Golgi (Itin *et al.,* 1995). Glycosylation is similarly important for sorting from the TGN (Scheiffele *et al.,* 1995; Gut *et al.,* 1998) and involves the ERGIC-53-related lectin VIP-36 (Fiedler and Simons, 1996), although no coat proteins have been established yet for this sorting event. Indeed, in

the case of apical transport of GPI-anchored proteins, coats have even been proposed not to be needed, with sorting being mediated instead by lipid-based sorting into glycosphingolipid ''rafts'' (Simons and Ikonen, 1997). In yeast, GPI anchors have shown to be necessary for the COPII-mediated sorting of Gas1p (Doering and Schekman, 1996), so a yeast ERGIC-53 homolog may be at work here. Truncation mutations in ERGIC-53 were shown to be the cause of decreased secretion of two highly glycosylated blood coagulation factors in certain hemophiliacs (Nichols *et al.,* 1998), and retention of ERGIC-53 in the ER decreases the ER export of the glycosylated lysosomal enzyme cathepsin C (Vollenweider *et al.,* 1998), supporting a role for ERGIC-53 in facilitating glycoprotein secretion. However, in both cases the secretion of most other glycoproteins appeared to be unaffected and no direct interactions of any of the lectins with their proposed cargoes have been found, so further work is clearly needed here. The mannose-6-phosphate receptors discussed earlier as transporting lysosomal enzymes from the TGN in AP-1/clathrin vesicles are obviously another example where glycosylation acts as a signal for transport, with the addition of this mannose-6-phosphate signal itself being dependent on prior recognition of a lysine-containing patch on the lysosomal enzymes (Kornfeld and Mellman, 1989).

Several clear sorting signals have been identified in ER resident proteins that direct their retrieval from the Golgi, including KKXX or KXKXX for membrane-bound proteins and KDEL for (mostly) soluble proteins (Teasdale and Jackson, 1996). A direct interaction has been demonstrated for KKXX-containing proteins with COPI *in vitro* (Cosson and Letourneur, 1994) and there is now *in vivo* evidence that this interaction may be physiological, as certain COPI mutants in yeast are defective in the retrieval of KKXX- and KDEL-bearing proteins from the Golgi (Banfield *et al.,* 1995; Letourneur *et al.,* 1994; Lewis and Pelham, 1996). While strongly implicating COPI in this transport step, it remains to be proven directly that KKXX proteins (other than ERGIC-53) are transported in COPI-coated vesicles. A novel ER retrieval motif has been identified that binds preferentially to the δ-COP subunit of coatomer, hence its name δL, for δ-COP ligand (Cosson *et al.,* 1998). It is present notably in the ER resident Sec71p, whose retrieval from the Golgi has been shown previously to be dependent on COPI (Sato *et al.,* 1997). That its critical feature is the presence of at least one aromatic residue (W/YxxxW/F/Y) underscores the functional similarity of δ-COP to the homologous μ chains of the clathrin adaptors that bind tyrosine motifs (discussed earlier).

Thus, although many data implicate coat proteins in the sorting and concentration of cargo, it is not clear if this determines the site of budding or if the coats in most cases are directly involved. Cargo proteins are

obviously not absolutely necessary for coat recruitment, but very probably modulate the rate of budding according to need.

2. Deformation of the Membrane

As well as cargo selection, another proposed role for coat proteins in vesicle budding is the physical deformation of the membrane into a bud and subsequent pinching off. This was first suggested on visualization of the regular hexagon and pentagon lattice surrounding purified clathrin-coated vesicles (Kanaseki and Kadota, 1969) and is supported by the ability of clathrin to self-assemble in the absence of membranes (Pearse, 1975; Ahle *et al.,* 1988) and the smooth increase in size of coated pits on incorporating more clathrin hexagons and pentagons, seen by deep etch electron microscopy (Heuser, 1980). Although this was disputed on finding that clathrin polymerization was not necessary for bulk-phase endocytosis (Cupers *et al.,* 1994), it is now evident that the cell can rapidly induce alternative clathrin-independent pathways of endocytosis to compensate (Lamaze and Schmid, 1995).

The strongest evidence implicating coat proteins directly in membrane deformation is the production of buds or vesicles from artificial protein-free acidic liposomes by the simple addition of either clathrin and adaptors alone, COPII components plus Sar1-GTP, or coatomer plus ARF-GTP (Takei *et al.,* 1998; Matsuoka *et al.,* 1998b; Spang *et al.,* 1998). Admittedly, the vesicles were rather deformed and the conditions far from physiological, but these experiments demonstrate that coats are capable of sculpting vesicles from larger membranes. The purified AP-3 complex has also been shown to drive synaptic vesicle budding from endosomal membranes, which is also dependent on ARF-GTP and other unidentified membrane proteins (Faúndez *et al.,* 1998). Membrane proteins, probably including cargo, may help control not only the size and shape of the vesicle, but also regulate the site and timing of vesicle formation, thus providing specificity to the budding event. Because these and other data (Fleischer *et al.,* 1994; Beck and Keen, 1991; Chang *et al.,* 1993) show that coat proteins can bind directly to certain phospholipids, coat proteins may be able to package specific lipids as well as proteinaceous cargo, although there is no evidence that coated vesicle lipid composition is different than that of the donor membrane. Preliminary results indicate that this is in fact not the case (see Nickel and Wieland, 1997), although electron micrographs show that the interleaflet space of the membrane of both COPI- and COPII-coated buds and vesicles appears thinner than that of the uncoated donor membranes (Orci *et al.,* 1996).

The main argument that has been raised against the coat proteins causing membrane deformation *in vivo* is that ARF-GTP is necessary not only for

coatomer recruitment, but also (at least in mammalian cells) for the activation of phospholipase D, which breaks down phosphatidylcholine in the membrane into negatively charged phosphatidic acid and choline (Brown *et al.,* 1993; Cockcroft *et al.,* 1994). The resulting net increase in negative charge and/or the different shape of the lipids could alter the local membrane fluidity and be the driving force for bud formation. The coat could therefore simply stabilize or facilitate formation of these buds through interaction with ARF rather than forming the bud per se (Ktistakis *et al.,* 1995). Indeed, cells with a high basal activity of phospholipase D appear not to need ARF at all for coatomer recruitment to Golgi membranes and their COPI vesicles appear to be ARF-free (Ktistakis *et al.,* 1996), although the difficulties in detecting ARF mean that stoichiometric levels may in fact still be present (Stamnes *et al.,* 1998). When ARF-GTP was omitted from the acidic liposome budding experiments mentioned earlier (Spang et al., 1998), coatomer was able to bind to the membranes but did not produce any vesicles. The main role for ARF could therefore simply be to prime the membrane for coatomer binding, in which case it may not need to enter COPI vesicles at all, and in fact has only ever been found there in the presence of the poorly hydrolyzable GTP analog GTPγS. However, phospholipase D activation cannot be the sole role of ARF, as disruption of the yeast phospholipase D gene has no effect on secretion (Waksman *et al.,* 1996) and the levels of phosphatidic acid (the product of phospholipase D activation) decrease rather than increase during budding (Stamnes *et al.,* 1998). Thus ARF probably has a dual role in facilitating coat binding through both membrane lipid modification and direct binding of the coat proteins.

We can conclude from all this that coat proteins are capable of providing the driving force for membrane deformation *in vitro,* but it is not yet clear to what extent this is true *in vivo.*

3. Interaction with the Targeting Machinery

In evaluating whether coat proteins might interact with the targeting or translocation machinery, one has to consider at what stage the coat dissociates from the vesicle and the distance that the vesicle has to travel. There is controversy as to how long a coated vesicle remains coated after detachment from the donor membrane under physiological conditions. If coats are only necessary to select cargo and/or deform the membrane into a vesicle, uncoating could occur immediately after pinching off, in which case a role in translocation would be unlikely. It is clear that a vesicle must uncoat at some point prior to fusion, as use of nonhydrolyzable GTP analogs to prevent uncoating leads to an accumulation of coated vesicles or buds that

cannot fuse with the target membrane (Orci *et al.,* 1989; Weidman *et al.,* 1993; Barlowe *et al.,* 1994).

For many transport steps, such as intra-Golgi transport, because the distance between the donor and the acceptor compartments is very small, a vesicle could simply diffuse to its target membrane and fuse relatively rapidly, making it difficult to assess the lifetime of the coat. Long-distance transport, such as TGN to plasma membrane (Lafont et al., 1994; Toomre et al., 1998), peripheral ER to Golgi (Presley *et al.,* 1997; Le Bot *et al.,* 1998), early to late endosome (Aniento *et al.,* 1993), and axonal transport (Hirokawa, 1993), occurs actively by motor proteins along microtubules and maybe also by actin filaments (Fath *et al.,* 1994; Maples *et al.,* 1997; Durrbach *et al.,* 1996). At least for the case of transport complex movement between the ER to the Golgi, it has been shown that the COPI coat can remain associated during microtubule-directed transport (Scales *et al.,* 1997; Shima *et al.,* 1999), raising the question as to whether coat proteins could play a role in targeting the vesicles via interaction with motors or the cytoskeleton (Lippincott-Schwartz *et al.,* 1995). Although the 110K β-subunit of COPI was originally identified as a microtubule-binding protein (Allan and Kreis, 1986), the antibody used to detect it cross-reacts with the microtubule-associated protein MAP2, and it also appears that coatomer can sediment nonspecifically under certain conditions, suggesting that β-COP is not a true microtubule-binding protein (R. Duden and T. E. Kreis, unpublished results). A link between intra-Golgi vesicles and microtubules is rabkinesin-6, a potential effector of the rab6 GTPase thought to regulate retrograde transport within the Golgi stack (Echard *et al.,* 1998). However, although COPI is likely to be the coat involved here (discussed earlier), a link between COPI and rab6/rabkinesin-6 has yet to be reported. No coat proteins have been unambiguously identified so far as mediating the other long-distance transport steps, so this issue remains unresolved.

Interestingly, the candidate TGN-vesicle protein p200 (Narula and Stow, 1995) has been shown to be a nonmuscle myosin II (Ikonen *et al.,* 1996; Müsch *et al.,* 1997), so interactions of these vesicles with the actin or Golgi spectrin-based cytoskeleton were predicted, although it was not clear whether the antibodies used in these studies were really specific (see Section VI for more details). The actin cytoskeleton can be remodeled by Rho-GTPases, leading to changes in the location and dynamics of early endosomes and inhibiting clathrin-dependent endocytosis (Murphy *et al.,* 1996; Lamaze and Schmid, 1995). Interestingly, Rho proteins can activate phospholipase D (as can ARF and protein kinase C, mentioned in Section V), perhaps explaining the observed stimulation of secretion by Rho in mast cells (Price *et al.,* 1995). Pan1p/Eps15 also appears to link endocytic membrane traffic to the actin cytoskeleton (Wendland and Emr, 1998). For

more details on membrane–cytoskeleton interactions, refer to Kreis *et al.* (1997) and Goodson *et al.* (1997).

4. Regulation of Docking

After the coated vesicle has been transported to its target membrane, the final possibility for a role of the coat proteins could be in the regulation of its docking. As mentioned earlier, coat proteins must be removed to permit exposure of the v-SNAREs to their cognate t-SNAREs in the target membrane. There is no convincing evidence that some of the coated buds presumed to be the precursors of coated vesicles are not in fact half-uncoated vesicles in the process of fusing with the target membrane. One could therefore speculate that the GTP hydrolysis on the small GTP-binding protein that recruited the coat may be regulated by interaction of the coat with some component of the docking machinery at the target membrane.

In the COPII coat, the Sec23p subunit is the GTPase-activating protein (GAP) for Sar1p, but does not act immediately, as vesicle formation is not initiated until after the Sec13p/Sec31p complex binds and GTP hydrolysis is not necessary for budding (Yoshihisa *et al.,* 1993; Salama *et al.,* 1993, 1997; Barlowe *et al.,* 1994). Thus further factors may be necessary to increase the hydrolysis rate and trigger uncoating. In addition, COPII vesicles formed in the presence of excess Sec23p/Sec24p are inhibited in fusion without being affected in Uso1p-mediated docking, suggesting that Sec23p/Sec24p may bind to v-SNAREs to prevent fusion (Barlowe, 1997).

Cross-linking of the COPI coat shows that β-COP, probably bound to δ-COP, binds to ARF-GTP (Zhao *et al.,* 1997; Pavel *et al.,* 1998), but unlike Sec23p for Sar1p, the GAP for ARF is not a component of the coatomer (Cukierman *et al.,* 1995). The Golgi localization of ARF-GAP suggests that its activity may be regulated to avoid coat shedding before vesicle formation is complete (Cukierman *et al.,* 1995). Indirect evidence for a role of coatomer in regulating docking is that the γ- and β'-COP genes, Sec21 and Sec27, respectively, interact genetically with the v-SNAREs Sec22, Bet1, and Bos1 and the activating rab protein of the latter, Ypt1, so coatomer may sequester the active SNARE complex (Newman *et al.,* 1990; Lian *et al.,* 1994; Duden *et al.,* 1994). At least for long-distance ER-to-Golgi transport, the COPI coat has been shown to remain at least partly bound to the transport complex membrane during its translocation (Scales *et al.,* 1997; Shima *et al.,* 1999).

Clathrin coats are intrinsically more stable than COP coats, so GTP hydrolysis by ARF (for AP-1; the factor that recruits AP-2 is not yet known) is not sufficient for uncoating: both the uncoating protein auxilin (Ungewickell *et al.,* 1995) and a heat shock-related ATPase hsc70 (Schloss-

man *et al.,* 1984; Greene and Eisenberg, 1990) are required, and the factors necessary for adaptor protein release still await discovery.

A possible candidate for a regulator of uncoating could be the rab proteins, which appear to be incorporated into vesicles at the budding stage, yet act at a later step prior to docking and fusion, perhaps in the activation of SNAREs (Søgaard *et al.,* 1994; Lian *et al.,* 1994), thus regulating SNARE complex formation (Hay and Scheller, 1997). A complex of the ER–Golgi rab1b protein with β-COP has been detected *in vitro* (Peter *et al.,* 1993) but was proposed to be necessary for budding rather than docking or fusion, although the converse is true of its yeast homolog Ypt1p, which shows a genetic interaction with Sec21/γ-COP (Søgaard *et al.,* 1994; Rexach *et al.,* 1994; Lupashin *et al.,* 1996; Newman *et al.,* 1990). Rab5-GTP has been shown to affect the docking and fusion steps of endocytosis through regulation by PI-3-kinase (Li *et al.,* 1995b), which, as discussed later (see Section V), also appears to regulate coat binding, thus budding and docking/fusion are closely coupled.

Another protein shown to regulate the docking of COPII vesicles is yeast Uso1p (Sapperstein *et al.,* 1996; Barlowe, 1997), whose *cis*-Golgi receptor may be a component of the TRAPP complex (Sacher *et al.,* 1998) and may regulate the Golgi fusion of COPII vesicles by regulating SNARE interactions. The mammalian equivalent of Uso1p is p115, which is thought to link p400/giantin on COPI vesicles to the *cis*-Golgi receptor GM130 (Sönnichsen *et al.,* 1998). The unrelated Sec6p/Sec8p/Sec15p complex is another candidate for regulating docking at the plasma membrane, but its mode of action remains unclear (TerBush and Novick, 1995; Hsu *et al.,* 1998). Thus there is no evidence yet that rabs or other proteins may control docking by regulating uncoating, but it remains a possibility because coated vesicles cannot fuse. Further data on coat proteins coupling budding to fusion are discussed in the tubular transport section (Elazar *et al.,* 1994; Taylor *et al.,* 1994).

B. The Maturation Model of Membrane Traffic

The maturation model of membrane traffic views all the membranous organelles not as stable compartments communicating via vesicular traffic, but as dynamic and constantly maturing. The role of coated vesicles in this model is to constantly retrieve material characteristic of earlier compartments of the pathway and to bring material characteristic of later compartments, allowing the donor compartment to mature into a ''later'' compartment. There is therefore no need for anterograde vesicular transport, as cargo destined for onward transport simply has to avoid being packaged into the retrograde vesicles. Because the roles of the coat proteins in matura-

tion are not fundamentally different to those in the vesicular transport, we will not discuss this model any further, but refer the reader to the following excellent reviews: Becker *et al.* (1995), Gruenberg and Maxfield (1995), Mironov *et al.* (1997), and Pelham (1998).

C. The Tubule Model of Membrane Traffic

The isolation of coated vesicles in cell-free or semi-intact cell systems and their visualization by electron microscopy is such compelling evidence as to their existence that an alternative mode of protein transport via tubular connections has largely been discounted. Nevertheless, electron microscopic studies have clearly illustrated the fact that many organelles in the biosynthetic and endocytic pathways are highly tubular in structure, and occasional connections between different compartments have also been observed (Farquhar and Palade, 1981; Rambourg and Clermont, 1997). Whereas the significance of these connections is unclear due to either the nonphysiological conditions used or their rarity, one possibility is that they serve to allow the transfer of protein and lipid between compartments. Instead of forming vesicles, coat proteins could simply act to gate the tubular connections by constriction and sort cargo into them. Alternatively, if the tubules detach from the donor compartment and fuse with the acceptor compartment, they could be considered as enlarged vesicles (Weidman, 1995), in which case the coat proteins would have the same roles as discussed earlier for vesicle formation. Such detaching tubules have been observed in living cells (Cooper *et al.,* 1990; Sciaky *et al.,* 1997), and although it was not clear whether tubule fusion was heterotypic in these cases, one can imagine that intercompartmental protein transport could occur along such connections, with coat proteins either regulating tubule formation, fusion site selection via regulation of SNARE exposure or driving their scission from the donor membrane.

While there is no evidence that COPII coats contribute to tubular traffic, there has been much more speculation as to the role of COPI in regulating such events. A role for β-COP in preventing tubule formation in the early biosynthetic pathway was initially proposed as a result of extensive studies using the fungal metabolite brefeldin A (BFA; Klausner *et al.,* 1992; Lippincott-Schwartz, 1993). These groups proposed that anterograde traffic is mediated by vesicles and retrograde traffic by tubule extension along microtubules because BFA treatment, whose earliest detected effect is the removal of β-COP from Golgi membranes (Donaldson *et al.,* 1990, 1991), results in the extension of tubules from the Golgi to the ER and the subsequent disappearance of the Golgi (Lippincott-Schwartz *et al.,* 1990). Overexpression of the KDEL receptor has the same effect (Hsu *et al.,*

1992), as this also leads to COPI dissociation from membranes due to overrecruitment of the ARF-GAP (Aoe *et al.,* 1997). In addition, GTPγS and AlF_4, which lock the COPI coat onto membranes (Donaldson and Klausner, 1994), prevent tubule formation and instead lead to an accumulation of COPI-coated vesicles presumed to be intermediates in anterograde transport. Having different modes of transport for forward and backward traffic is an attractive model for balancing membrane lipid flow without jeopardizing cargo transport, as spherical anterograde vesicles would contain a relatively large volume of cargo per unit area of membrane, whereas long thin tubules would ensure retrieval of the same area of membrane without entrapping much soluble cargo, which would be limited to the smaller amount of misfolded cargo or escaped ER proteins compared to that destined for anterograde flow (Lippincott-Schwartz, 1993). There is still no direct evidence, however, that BFA-induced retrograde tubules reflect normal retrograde traffic, and an alternative explanation is that the removal of COPI by BFA exposes v-SNAREs to t-SNAREs in adjacent compartments, leading to nonphysiological fusion (Elazar *et al.,* 1994), hence COPI can be viewed as a means of coupling the budding of vesicles to subsequent fusion. Removal of coatomer by ARF depletion (Taylor *et al.,* 1994) or by lowering ATP levels (Cluett *et al.,* 1993) similarly showed that intra-Golgi transport may occur by tubulation and fusion of Golgi membranes even in the absence of BFA, thus tubulation may be an exaggerated form of budding, normally suppressed by coat proteins. This may be analogous to the long tubular invaginations formed at the plasma membrane when dynamin is prevented from pinching off clathrin-coated buds (Damke *et al.,* 1994). In this respect it is noteworthy that the electron micrographs illustrating the strongest evidence to date for a role of COPI in bidirectional intra-Golgi vesicular transport reveal many more coated buds (suppressed tubules?) than coated vesicles (Orci *et al.,* 1997). Coat proteins could simply act as a mechanical barrier to fusogenic tubule projection, perhaps by preventing interactions with motor proteins necessary for tubule extension along microtubules (Lippincott-Schwartz *et al.,* 1995; Cole and Lippincott-Schwartz, 1995).

While no coat complex has been fully characterized yet at the molecular level (see Section VI and also Traub and Kornfeld, 1997), coated buds have been visualized on the highly tubular TGN (Griffiths *et al.,* 1995; Narula and Stow, 1995; Ladinsky *et al.,* 1994; Simon *et al.,* 1996). High voltage electron microscopy tomography also reveals that the TGN is composed of morphologically distinct domains, which could be due to proteins being sorted into tubules prior to or instead of vesicle budding (Ladinsky *et al.,* 1994). Coat proteins or distinct lipid microdomains (rafts; Simons and Ikonen, 1997) could segregate cargo into such tubular regions of the TGN and/or regulate their subsequent breaking off as intact tubules, break-

ing up into smaller vesicle sized units, or even transient extension to fuse with the target organelle. Interestingly, post-TGN transport intermediates visualized with VSV-G-GFP appear more tubular than their ER–Golgi transport complex counterparts (Toomre *et al.,* 1998).

The endosomal system is also highly tubular in many cell types, especially the recycling endosomes (Geuze *et al.,* 1983; Hopkins *et al.,* 1990; Marsh *et al.,* 1986). Recycling receptors have been shown to be more concentrated in the tubular elements of recycling endosomes (Geuze *et al.,* 1987), and coat proteins may be involved in the segregation into tubules and/or the removal of tubule contents, as discussed for the TGN, leaving the less tubular body of the endosome to either bud carrier vesicles destined for the next compartment (Griffiths and Gruenberg, 1991) or to mature into the next compartment (Murphy, 1991). Clathrin, AP-3, and COPI-related coats have been localized to endosomes (Stoorvogel *et al.,* 1996; Whitney *et al.,* 1995; Aniento *et al.,* 1996; Dell'Angelica *et al.,* 1998), and clathrin can even be recruited to lysosomes *in vitro* (Traub *et al.,* 1996), but the budding of coated vesicles has not yet been shown except in the case of AP-3-dependent synaptic vesicle formation (Faúndez *et al.,* 1998). One mode of endocytic transport visualized in living cells appears to involve ''boluses'' of material moving in a peristaltic fashion along tubular connections (Hopkins *et al.,* 1990), so whether coat proteins may play a role in constricting the tubule behind such boluses remains to be determined. Indirect evidence that this may be the case is the observation that COPI depletion or degradation of ε-COP inhibits the formation of both endocytic carrier vesicles from early endosomes and of their internal membranes, and disrupts targeting of proteins and fluid phase markers to late endosomes, whereas no coat has been detected yet on these vesicles (Aniento *et al.,* 1996; Gu *et al.,* 1997; Daro *et al.,* 1997). Because COPI removal by BFA (Lippincott-Schwartz *et al.,* 1991) or ε-COP degradation (Gu *et al.,* 1997) results in the tubulation of endosomes, a structural or sorting role for COPI via the regulation of tubule formation in the endocytic pathway appears more likely.

V. Regulation of Membrane Traffic through Coat Proteins

Membrane traffic must be regulated in response to the external environment as well as to the needs of the cell itself, and cell signaling pathways, including lipid metabolism, kinases, and heterotrimeric G-proteins, are involved in its regulation, often at the level of coat protein recruitment.

There is a growing body of evidence for the role of lipid metabolism in vesicle budding other than the potential regulation by ARF and phospholipase D already discussed. Phosphatidylinositols (PtdIns), their phosphorylated derivatives, phosphoinositides (PIs), and inositol polyphosphates (InsPPs) are among the lipids that play a major role at various steps of vesicle formation (Liscovitch and Cantley, 1995; De Camilli *et al.,* 1996; Roth and Sternweis, 1997). The identification of the Vps34 gene, required for yeast protein sorting in late Golgi to vacuole transport (Herman and Emr, 1990), as the catalytic domain of phosphatidylinositol 3-kinase (PI 3-kinase: Schu *et al.,* 1993) suggests that vesicular transport may be regulated by intracellular signaling and be able to respond to external stimuli. One suggestion is that the localized production of PtdIns-3-phosphate products by Vps34p/PI 3-kinase may recruit coat proteins (Stack *et al.,* 1995), perhaps through stimulation of the nucleotide exchange required for ARF1 recruitment to membranes (Terui *et al.,* 1994). In addition, activated ARF1, in concert with PtdIns(4,5)P_2, activates phospholipase D (PLD), which in turn activates ARF by generating phosphatidic acid (PA). PA then activates PtdIns (4,5)P_2 kinase to generate additional PtdIns(4,5)P_2 and both stimulate the activity of an ARF GAP (Randazzo and Kahn, 1994); thus there is a feedback loop that ensures rapid coat recruitment. In the case of TGN to vacuole transport, it is therefore likely that the AP-1 adaptor will bind to PtdIns. Because ARF-1 also recruits COPI and AP-3 to their donor membranes (Donaldson *et al.,* 1992a; Faúndez *et al.,* 1998), PI 3-kinase may indirectly regulate the recruitment of these coats too.

PI 3-kinase is also involved in endocytosis and transport to endosomes (Shepherd *et al.,* 1996), as evidenced by the fact that the PI 3-kinase inhibitor wortmannin inhibits early endosome fusion (Li *et al.,* 1995b), missorts lysosomal cathepsin D (Brown *et al.,* 1995; Davidson, 1995), alters the pathways of transferrin receptor (Spiro *et al.,* 1996) and mannose-6-phosphate receptor (Martys *et al.,* 1996), and decreases downregulation of the PDGF receptor (Joly *et al.,* 1994). That wortmannin does not completely block endocytosis may be explained by the discovery of a wortmannin-resistant PI 3-kinase (Feng and Pawson, 1994). One mechanism for the action of PI 3-kinase in regulating endocytosis may be the binding of its product PtdIns-3-phosphate to the α-subunit of the endocytic clathrin adaptor AP-2 (Beck and Keen, 1991), which has been shown to enhance both AP-2 and clathrin binding to tyrosine-based endocytic motifs (Rapoport *et al.,* 1997). α-adaptin may also be recruited through binding other InsPPs, such as PtdIns(4,5)P_2 and PtdIns(1,4,5)P_3 (Beck and Keen, 1991; Gaidarov *et al.,* 1996). It has been shown more recently that the early endosome autoantigen EEA1 is an effector of rab5 and that its membrane association requires PI_3P and PI 3-kinase activity (Simonsen *et al.,* 1998), consistent with the earlier observation that wortmannin causes the release of EEA1 from early endo-

somes (Patki *et al.,* 1997). EEA1 thus provides a direct link between PI 3-kinase function and the regulation of early endosome fusion by rab5.

Further links between phosphorylated PtdIns products or inositol polyphosphates and endocytosis are that PtdIns(4,5)P_2, but not PtdIns 4-P (Lin and Gilman, 1996), stimulates the GTPase activity of dynamin. Interestingly, PtdIns(4,5)P_2, Ins(1,4,5)P_3, and Ins(1,3,4,5)P_4 are substrates for the 5-phosphatase synaptojanin, a nerve terminal protein (McPherson *et al.,* 1996) that has been proposed to decrease the concentration of PtdIns(4,5)P_2 in coated vesicles (McPherson *et al.,* 1996). Synaptotagmin binds both Ins-PPs and the AP-2 complex via its C2B domain (Fukuda *et al.,* 1995; Li *et al.,* 1995a), antibodies against which block synaptic vesicle recycling (Fukuda *et al.,* 1995), illustrating the importance of this domain in endocytosis. Thus, PIs and Ins polyphosphates recruit, activate, and regulate not only coat proteins, but also other factors required for the formation of different coated vesicles.

PI 3-kinase is also necessary for constitutive budding from the TGN (Jones and Howell, 1997), as its 85K regulatory subunit was shown to be the p62/rab6 complex, discovered previously as a complex associated with TGN vesicles (Jones *et al.,* 1993). This subunit is a target for the GTP-bound forms of Rac and Cdc42 (Zheng *et al.,* 1994) and PI 3-kinase itself activates nucleotide exchange on Rac (Hawkins *et al.,* 1995), thus there is a feedback loop connecting lipid kinases with GTPases involved in the regulation of the actin cytoskeleton necessary for efficient exo- and endocytosis. Furthermore, the Sec14 gene product (PEP3, priming of exocytosis protein 3 in mammals), also required for budding from the TGN, is a phospholipid transferase recognizing both phosphatidylinositol and phosphatidylcholine and may act to balance the levels of these two phospholipids in the Golgi (Alb *et al.,* 1996). Data suggest that the Sec14 defect is related to excessive consumption of diacylglycerol (DAG), a lipid essential for budding, since it can be suppressed by a mutant in Sac1p, which normally increases DAG production via another pathway (Kearns *et al.,* 1997).

Interestingly, PI 3-kinase itself has been shown to bind directly to the src-phosphorylated platelet-derived growth factor β receptor (PDGFβR), along with the COPI subunits α and β', which bind independently of phosphorylation (Hansen *et al.,* 1997). However, the significance of the COPI interaction remains unclear, as (1) a mutation in the PDGFβR that inhibited COPI binding had no effect on PDGF's signaling pathways and (2) PDGFβR and PI 3-kinase are cointernalized in clathrin-coated, not COPI-coated vesicles (Kapeller *et al.,* 1993).

Another enzyme implicated in the regulation of membrane traffic through coat proteins is protein kinase C (PKC), which has long been known to be stimulated by DAG, one of the products of the action of phospholipase C on PtdIns(4,5)P_2 as a result of cell surface receptor activation, and hence

has an established role in the cell-signaling cascade (Parker, 1994). PKC has been shown to increase secretion and membrane binding of ARF and coatomer (De Matteis *et al.,* 1993). PKC itself is recruited from the cytosol to membranes, including Golgi membranes, on activation (Saito *et al.,* 1989; Lehel *et al.,* 1995), which could be a potential means of regualting budding sites. The ε isoform of PKC appears to bind to the Golgi via β'-COP, perhaps via their WD-40 motifs (Csukai *et al.,* 1997). In addition, β-COP has three consensus PKC serine phosphorylation sites (Duden *et al.,* 1991) and is phosphorylated on multiple serines *in vivo* (Sheff *et al.,* 1996), although it has not yet been shown whether this is mediated by PKC. If it is, it may explain the mechanism by which PKC increases transport between the ER and Golgi (Fabbri *et al.,* 1994). Constitutive budding from the TGN also requires protein kinase C (Simon et al., 1996). Like ARF, PKC also stimulates phospholipase D, and the activity of a BFA-sensitive ARF-like protein is required for the coating of TGN vesicles (Sabatini *et al.,* 1996).

Heterotrimeric G-proteins have also been implicated indirectly in many steps of membrane traffic, including ER to Golgi, intra-Golgi, TGN to plasma membrane (both apical and basolateral), exocytosis of regulated secretory granules, endocytosis, and transcytosis (Stow and Heimann, 1998). Many studies have utilized AlF_4 as a specific activator of heterotrimeric (but not small) GTP-binding proteins (Kahn, 1991), ribosylating toxins, or compounds which affect or mimic heterotrimeric G-proteins, but caution is needed in interpreting such results, as they have many diverse effects in the cell (Helms, 1995). The first such study showed that AlF_4 inhibited intra-Golgi transport (Melançon *et al.,* 1987), and subsequently the G-protein was shown to be the Golgi-localized Gαi-3, as its overexpression inhibits the transport of heparin sulfate proteoglycan through and from the Golgi (Stow *et al.,* 1991). One of the membrane trafficking steps thought to be regulated by heterotrimeric G-proteins is budding, as the formation of constitutive and regulated secretory vesicles from the TGN is inhibited by a Gαi subunit of a trimeric G-protein (Barr *et al.,* 1991) and is stimulated by Gαs subunits (Leyte *et al.,* 1992). AlF_4 recruits COPI and prevents its release from Golgi membranes by preventing GTP hydrolysis on ARF (Donaldson *et al.,* 1991; Finazzi *et al.,* 1994), although the mechanism of this is not yet understood. Interestingly, G$\beta\gamma$ subunits prevent coatomer binding to membranes (Donaldson *et al.,* 1991) and also cause vesiculation of the Golgi complex (Jamora *et al.,* 1997). ARF can also interact with the Gα subunit of heterotrimeric G-proteins (Colombo et al., 1995), which is also consistent with a role for heterotrimeric G-proteins in vesicle formation via coat recruitment.

Interestingly, the β subunit of heterotrimeric G-proteins contains WD-40 motifs also found in α- and β'-COP and Sec13p/Sec31p of the COPII coat (Neer *et al.,* 1994; Letourneur *et al.,* 1994; Faulstich *et al.,* 1996; Stenbeck

et al., 1993; Harrison-Lavoie *et al.,* 1993; Pryer *et al.,* 1993; Salama *et al.,* 1997), but it is not yet clear whether this has anything to do with the membrane binding of the coats or is just a means of protein–protein interactions. Binding of AP-2 and p200/myosin to TGN membranes are similarly regulated by heterotrimeric G-proteins (Robinson and Kreis, 1992; de Almeida *et al.,* 1993). Other roles for heterotrimeric G-proteins in membrane traffic include receptor-mediated exocytosis (Vitale *et al.,* 1993; Ahnert Hilger *et al.,* 1994; Lang *et al.,* 1995), endosomal fusion (Colombo *et al.,* 1994), transcytosis (Bomsel and Mostov, 1993), and protein packaging (Pimplikar and Simons, 1993). Because heterotrimeric G-proteins are well established as signal transducers at the plasma membrane (Neer, 1995), the regulation of budding by their various subunits provides an attractive means of adjusting secretion in response to extracellular stimuli.

The dynamin family of GTPases also regulates vesicle budding, although maybe not directly through coat proteins. Dynamin was first implicated in neuronal endocytosis by virtue of its homology to the *Drosophila shibire* product (van der Bliek and Meyerowitz, 1991), whose mutation causes rapid inhibition of synaptic vesicle recycling at a nonpermissive temperature. The observed accumulation of collared pits suggested that dynamin was necessary for the pinching off of vesicles, which was supported by the induction of similar dynamin-coated collars by GTPγS (Takei *et al.,* 1995; Hinshaw and Schmid, 1995). GTP hydrolysis is necessary for clathrin-coated vesicle scission (Carter *et al.,* 1993), and a dominant negative GTP-binding deficient mutant of dynamin is not concentrated at bud necks, leading to the following working model: dynamin is recruited uniformly to the lattice of clathrin-coated pits in its GDP form, GTP/GDP exchange leads to its redistribution into a constricted coated pit structure around the base of the pit, and GTP hydrolysis allows scission, possibly triggered by the GTPase activity of dynamin (Warnock and Schmid, 1996; De Camilli *et al.,* 1995; Takei *et al.,* 1995; Hinshaw and Schmid, 1995). Direct evidence for a mechanical role of dynamin in tubulation and vesiculation of acidic phospholipid liposomes on GTP hydrolysis has now been provided (Sweitzer and Hinshaw, 1998; Takei *et al.,* 1998), but other aspects of this model have recently been challenged.

There are now three classes of dynamin (Dyn1, Dyn2, and Dyn3), with differing tissue distributions (Nakata *et al.,* 1993; Cook *et al.,* 1994; Sontag *et al.,* 1994). In addition, a novel dynamin-like protein, called Dymple or DLP1, has been identified (Kamimoto *et al.,* 1998; Yoon *et al.,* 1998). Whereas epitope-tagged Dymple/DLP1 localizes to the Golgi, the endogenous protein is mostly cytosolic and associates with cytoplasmic vesicles and tubules of the endoplasmic reticulum, providing evidence that dynamin-like proteins may also be implicated in budding events in the early secretory pathway.

A dynamin-related protein may also be involved in budding from the Golgi, as the N-terminal domain of dynamin shares homology with the yeast vacuolar protein, Vsp1p, which is involved in protein sorting from the Golgi apparatus (Rothman *et al.,* 1990; Vater *et al.,* 1992; Wilsbach and Payne, 1993), and antibodies against conserved regions of the dynamin family label a dynamin-like protein on the Golgi (Henley and McNiven, 1996). In addition, immunodepletion of dynamin or anti-Dyn2 antibodies inhibits the budding of both constitutive exocytotic and clathrin-coated vesicles from the TGN (Jones *et al.,* 1998), and electron microscopy shows that Dyn2 localizes to the TGN (Maier *et al.,* 1996). However, whereas dynamin colocalizes with clathrin and AP-2 at the rims of plasma membrane coated pits, no such colocalization with AP-1 in the TGN has been reported yet. The ear domain of α-adaptin of AP-2 can bind dynamin directly *in vitro* (Wang *et al.,* 1995), but other experiments suggest that dynamin is recruited indirectly to α-adaptin through the SH3 domain of amphiphysin (David *et al.,* 1996). Thus, although dynamins may well be involved in the regulation of vesicle budding, it is not yet clear whether the different dynamins are recruited by the respective coat proteins.

There are thus many links among lipid metabolism, GTP-binding proteins, and cell signaling pathways in the regulation of membrane traffic through coat proteins.

VI. Emerging Families of Coat Proteins

We have already discussed the four kinds of vesicle-associated coat protein complexes that have been characterized so far: the clathrin coat with its AP-1 and AP-2 adaptors, the AP-3 complex, the COPI, and the COPII complexes. It was proposed a long time ago that most or all of the vesicular transport steps in the cell would be mediated by coat proteins. It is now clear that the adaptors AP-1, AP-2, and AP-3 are closely related not only in function, but also partially in sequence and so can be considered a subfamily within the overall ''family'' of coat proteins. Three of the COPI subunits (β, δ, and ζ) show limited homology to the adaptors of the corresponding sizes, whereas the other COPI subunits and COPII share no significant sequence identity other than WD-40 repeats. Nevertheless, their probable functions in cargo sorting and potential membrane deformation are clearly similar. Each of these coats has been associated with different pathways, but there are many other vesicular transport events for which no coat proteins have yet been clearly characterized, suggesting that other members of the overall coat family await identification.

Indeed, μARP-1 and its 60% identical isoform, μARP-2, have been identified as ubiquitous relatives of μ1 and μ2 adaptins (with 27–31% identity; Wang and Kilimann, 1997). μARP-2 has been proposed to be the μ4 subunit of a newly characterized AP-4 complex (Dell'Angelica *et al.,* 1999; J. Hirst and M.S. Robinson, personal communication), whose role remains to be determined. μARP-1 shows very weak homology to the newly identified TIP47 (21% over only one-fourth of the protein; Díaz and Pfeffer, 1998), which has been proposed to act as mannose-6-phosphate receptor for endosome to TGN transport, so it is tempting to speculate that an AP-4 like complex may mediate this retrograde transport step. However, neither TIP47 nor μARP-1 appear to share any homology with the candidate yeast endosome–Golgi retromer coat complex (see later; Seaman *et al.,* 1998). This may not be unexpected, as yeast do not have mannose-6-phosphate receptors, and indeed distinct poorly characterized mammalian homologs of retromer proteins do exist, the best known being SNX1 (Vps5p homolog), which binds to EGF receptors (Kurten *et al.,* 1996).

In addition, a novel ubiquitously expressed isoform of γ-adaptin (with >60% identity) has been identified and termed γ2-adaptin (Lewin *et al.,* 1998; Takatsu *et al.,* 1998). Although localized to the Golgi complex, γ2-adaptin is found on different vesicles to γ-adaptin and, unlike γ-adaptin, is resistant to BFA treatment. However, two-hybrid analysis shows that like γ-adaptin, γ2-adaptin can interact with σ1 (as well as a closely related σ1B isoform; Takatsu *et al.,* 1998), and so it may regulate a distinct trafficking step without being part of a completely novel coat.

Ultrastructural studies have provided evidence that proteins related to known members of the different coat subfamilies are implicated in endocytosis and in transport from the TGN. COPI-related proteins have been shown to associate with endosomes and are involved in endosome function (Whitney *et al.,* 1995; Aniento *et al.,* 1996). In addition, the ε-COP^{ts} ldlF mutant is defective in endocytosis as well as secretion (Guo *et al.,* 1994, 1996, 1997; Daro *et al.,* 1997). A novel class of clathrin-coated vesicles budding from endosomes has also been detected (Stoorvogel *et al.,* 1996) and is proposed to have a function in recycling from the endosomes via the plasma membrane, although no direct evidence for this was shown. Subsequent work has implicated AP-3 as the adaptor involved here (Dell'Angelica *et al.,* 1998), although this is still controversial (Simpson *et al.,* 1996; Faúndez *et al.,* 1998). In yeast, a new candidate for a novel endosomal coat is the "retromer," a complex of the Vps26, Vps29, Vps35, Vps5, and Vps17 gene products that is proposed to mediate recycling of the carboxypeptidase Y receptor, Vps10p, from the prevacuolar endosome to the TGN (Seaman *et al.,* 1998). Vps5p exhibits self-assembly properties *in vitro* and the epitope-tagged protein localizes to specific sites on the prevacuolar endosome, but analysis of the endogenous complex awaits

the generation of suitable antibodies. Another yeast complex containing Vps41p/Vam2 and Vps39p/Vam6 has been detected on vacuoles and may be involved in the alkaline phosphatase transport pathway from the Golgi. Intriguingly, both proteins share a ~100 amino acid motif with ret1p/α-COP and the clathrin heavy chain, chc1p, giving rise to the speculation that they may be part of another new coat complex (Conibear and Stevens, 1998).

From the TGN, proteins are sorted to endosomes, the cell surface, or recycled to the ER or earlier compartments of the Golgi (Traub and Kornfeld, 1997). COPI-coated vesicles have been seen budding from the TGN, but it is not known if these mediate retrograde transport to earlier Golgi compartments or the ER or represent another related form of COPI involved in transport to the plasma membrane (Griffiths *et al.,* 1995). Novel forms of ζ- and γ-COP may also function in a new form of coatomer (Whitney and Kreis, 1998), and whether the other COP subunits of this complex are identical or only related to coatomer remains to be determined.

A lace-like coat, whose structure differs significantly from that of the coatomer and the clathrin coats, has been visualized at the TGN (Ladinsky *et al.,* 1994), and several cytosolic proteins have been identified that are candidates for components of this coat. One is the coat-like protein, p200, which was found to behave like β-COP in its sensitivities to BFA and GTPγS, yet is associated with different TGN buds and vesicles to both COPI and clathrin (Narula *et al.,* 1992; Narula and Stow, 1995; de Almeida *et al.,* 1993). However, cloning of this protein revealed it to be a nonmuscle myosin II (Ikonen *et al.,* 1996; Müsch *et al.,* 1997), although it was not clear in these studies whether the antimyosin antibodies were really specific. If p200 really is myosin, it should perhaps no longer be viewed as a coat, despite its β-COP-like properties (Narula *et al.,* 1992), and indeed the latest findings indicate that the anti-p200 antibody (AD7) cross-reacts with native coatomer, although there is still controversy as to its role in TGN-vesicle budding (Simon *et al.,* 1998; Ikonen *et al.,* 1996; Müsch *et al.,* 1997).

A more likely candidate is p230, which is found on different TGN buds and vesicles to p200, is removed from the membrane by BFA with slower kinetics than p200 and β-COP, and is stabilized on membranes by GTPγS (Gleeson *et al.,* 1996).

Another candidate is the cytosolic p62/rab6 complex, which is associated with TGN38/41, a protein that has cycles between the plasma membrane and TGN, as immunodepletion of either p62 or rab6 inhibits the budding of exocytic transport vesicles (Jones *et al.,* 1993). However, this complex has been shown to be the regulatory subunit of PI-3 kinase, so it perhaps acts more as a regulator of membrane lipids and thus coat recruitment than as a coat protein itself (Jones and Howell, 1997).

In yeast, the COPII subunit Sec13p has been implicated in the nitrogen-regulated TGN–plasma membrane transport of Gap1p, as well as in ER–Golgi transport, so there may be another COPII-related coat to be discovered (Roberg *et al.,* 1997b). However, other genes affecting this pathway (Lst4, Lst7, and Lst8) appear not to be homologous to other COPII components, but it remains to be determined whether they are components of this potentially new coat (Roberg *et al.,* 1997a). There is also another yeast homolog of Sec24p that remains to be fully characterized, so it is not yet clear whether it forms part of this novel TGN coat or simply selects different cargo for incorporation into the ER COPII coat. Likewise, in mammalian cells there are at least two homologs of Sar1p (Sar1a and Sar1b; Kuge *et al.,* 1994), two of Sec23p (Sec23a and Sec23b; Paccaud *et al.,* 1996), and four of Sec24p (hSec24a–d; Pagano *et al.,* 1999), which were discussed in the COPII section.

Because of the relatedness of the different coat components within each coat subfamily, a powerful approach to the identification of new family members is to search the database for homologs of known coats, as has been successful for the identification of both mammalian and yeast AP-3 complexes (Simpson *et al.,* 1997; Dell'Angelica *et al.,* 1997; Cowles *et al.,* 1997a). Examination of the developing human cDNA databases and the completed yeast database has already indicated that there is at least one other novel AP-like complex, the AP-4 complex (Dell'Angelica *et al.,* 1999; Cowles *et al.,* 1997a), and as more genes are sequenced, the potential for discovering other new coats will increase.

VII. Conclusions and Perspectives

The last few years have seen major advances in our understanding of the roles of coat proteins in membrane traffic. The discovery of the p24 family and other potential coat–cargo adaptor proteins implicates coat proteins other than the well-established clathrin adaptors in cargo selection. Old and new coats are being characterized rapidly at the molecular level, with different functions now being ascribed to different coat subunits. The ability to produce coated vesicles from pure lipid membranes with just coat components similarly implicates other coat proteins than the previously established clathrin in membrane deformation. A radical change of view is that membrane lipids are now realized to be not only structural, but also key regulatory components of the coat recruitment and membrane deformation machinery, although the sequence of events remains to be clarified. Advances in electron and light microscopy techniques have shown that tubules may be more important to membrane traffic than popularly thought, and visual-

ization of secretory traffic in living cells using GFP and analysis of SNAREs have also revived interest in the maturation model of Golgi transport. The completion of the yeast genome and the expanding mammalian databases have also been instrumental in speeding up the characterization of new coat proteins and other proteins regulating membrane traffic.

Through the complementary use of yeast genetics, *in vitro* assays, semipermeabilized cell systems, and *in vivo* analyses, a better picture of membrane traffic is emerging. The consensus seems to be that coat protein functions are more conserved than the coat subunits themselves. With the new assays that have been developed for vesicle budding from the TGN and endosomes and the growing DNA databases, it should not be long before new coat proteins involved in these trafficking events are also identified.

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Regulation of Monoamine Receptors in the Brain: Dynamic Changes during Stress

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Monoamine receptors are membrane-bound receptors that are coupled to G-proteins. Upon stimulation by agonists, they initiate a cascade of intracellular events that guide biochemical reactions of the cell. In the central nervous system, they undergo diverse regulatory processes, among which are receptor desensitization, internalization into the cell, and downregulation. These processes vary among different types of monoamine receptors. α_2-Adrenoceptors are often downregulated by agonists, and β-adrenoceptors are internalized rapidly. Others, such as serotonin$_{1A}$-receptors, are controlled tightly by steroid hormones. Expression of these receptors is reduced by the "stress hormones" glucocorticoids, whereas gonadal hormones such as testosterone can counterbalance the glucocorticoid effects. Because of this, the pattern of monoamine receptors in certain brain regions undergoes dynamic changes when there are elevated concentrations of agonists or when the hormonal milieu changes. Stress is a physiological situation accompanied by the high activity of brain monoaminergic systems and dramatic changes in peripheral hormones. Resulting alterations in monoamine receptors are considered to be in part responsible for changes in the behavior of an individual.
KEY WORDS: α_2-Adrenoceptors, β-Adrenoceptors, Adrenergic, Serotonin, 5HT$_{1A}$-receptors, Stress, Tupaia, Downregulation.

I. Introduction

The brain of an organism has two imperative functions: to regulate its physiology and to guide its behavior. Examples for important physiological processes that are controlled by the brain are the regulation of blood pressure and respiratory rhythm (respective control centers are located in

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the hypothalamus and the brain stem) and the regulation of the overall metabolic rate of the body, which is, among others, mediated by peripheral hormones such as cortisol and adrenaline. Brain centers that regulate release of these hormones from the adrenal gland are located in the hypothalamus and the hippocampus. The central nervous control of behavior is especially important in challenging situations that could be life-threatening if the organism does not react in the appropriate way. Under such circumstances, sensory systems and the motor system cooperate to carry out the adequate physical action (e.g., flight in view of a predator). Other systems modulate processes leading to these actions, e.g., behavioral reactions related to emotional events are either facilitated or inhibited by the limbic system [phylogenetically old brain regions (hippocampal formation and cingulate cortex, amygdala, hypothalamus, septum, and nucleus accumbens) that regulate emotions] with the amygdala playing a prominent role.

Central nervous regulatory processes are carried out by neurons. These cells transfer signals to other cells by releasing neurotransmitters that bind to specific receptors on the target cell. Such neurotransmitters can elicit a biochemical and/or electrical signal and thus evoke a response in the target neuron to be either active or silent. While the fast information transfer in the brain is carried out by amino acid transmitters such as glutamate and γ-amino butyric acid, which convey their signals within milliseconds to the targets, there are also transmitters that induce slower neuronal reactions. Among these ''slow neurotransmitters'' are monoamines that stimulate the monoaminergic receptors (e.g., adrenergic, serotonergic, and dopaminergic receptors). Via the slow mechanisms of signal transmission, which involve the functioning of intracellular second-messenger systems, monoamines modulate neuronal activity, meaning that they influence the responses to ''fast neurotransmitters'' such as glutamate. Although concentrations of monoamines in the brain are much lower than those of amino acid transmitters (catecholamine concentrations are in the nanomolar range, whereas glutamate is in the micromolar range), the slow transmitters play an important role in controlling many physiological and behavioral processes, especially those related to emotional events.

In the course of an individual's life, slow mechanisms of monoamine neurotransmission can be subjected to considerable changes. These may be related to developmental alterations in the expression of genes that encode elements that take part in monoamine neurotransmission, to changes in hormones or other compounds that regulate neurotransmission, or to lifetime periods accompanied by extensive activation of the systems involved. Challenging situations that induce the well-known physiological reactions characterized as stress response can lead to considerable changes in central nervous monoamine systems. Brain mechanisms that accompany stress prepare body and mind to react adequately to stressful stimuli in terms of physiology and behavior (e.g., increases in blood pressure are

necessary to supply the muscles with enough energy if flight is the only life-saving reaction in view of a predator). Both types of reactions, physiological and behavioral, are accompanied by an activation of the central nervous monoamine systems. If stressful situations occur repetitively over a longer time period, thus inducing chronic stress, the central nervous monoamine system changes its reactivity to the stimuli. This plasticity of the central nervous monoamine system is a prerequisite for long-term adaptation or maladaptation during periods of stress.

In recent years, the rapid development of molecular biology techniques and the expanding field of research on molecular structures of mammalian cells led to great progress in understanding the chemistry of regulatory mechanisms in monoamine systems. This review gives an overview of regulatory processes that take place in mammalian monoamine systems and describes their presumptive relation to physiology and behavior. Because the plasticity of monoamine receptors is especially apparent during stress, the last part of the review focuses on monoamine receptor changes during chronic stress.

II. Monoamines

Biogenic amines include the catecholamines dopamine, noradrenaline, and adrenaline, and the indolamine serotonin (5-hydroxytryptamine, 5HT). The biosynthesis of monoamines takes place as the stepwise conversion of amino acids in the presence of catalyzing enzymes. The catecholamines are derived from the amino acid L-tyrosine, which is converted into L-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (Hökfelt *et al.,* 1984). A second enzyme, aromatic amino acid decarboxylase, converts DOPA into dopamine, which can be further converted to noradrenaline (by the enzyme dopamine β-hydroxylase) and to adrenaline [by phenylethanolamine-*N*-methyltransferase (PNMT)]. The biosynthesis of noradrenaline was first demonstrated by Blaschko (1939). The presence of adrenaline in the central nervous system was first suggested by Vogt (1954). The following paragraphs describe the noradrenergic, the adrenergic, and the serotonergic system briefly before summarizing current knowledge on α_2-adrenergic receptors (α_2-ARs), β-adrenergic receptors (β-ARs), and serotonin$_{1A}$-receptors ($5HT_{1A}$-receptors) as examples of the respective receptor systems. For a review of other serotonin receptors, see Uphouse (1997). A description of the dopaminergic receptor system is beyond the scope of this review (Mansour and Watson, 1995).

A. Noradrenaline and Adrenaline

Neurons that synthesize and release noradrenaline (NA) from their nerve terminals are only present in the brain stem, i.e., in the pons and the medulla

oblongata, where they form clusters of cell bodies designated as groups A1 to A7 (Dahlström and Fuxe, 1964; Moore and Card, 1984; Nieuwenhuis, 1985). The most prominent nucleus (cluster of cells forming a neuroanatomical entity), the locus coeruleus (LC), is located in the pons and comprises groups A6 and A4, and scattered neurons that overlap with group A7, the subcoeruleus. Other pontine noradrenergic centers are A5 and A7. Medullary noradrenergic nuclei are A1 (in the ventrolateral medulla), A3 (dorsal to the olivary complex; this group has only been observed in rats, not in primates), and A2 (the solitary tract nucleus) in the dorsomedial medulla. Noradrenergic neurons project to many regions of the brain, and their processes form four main pathways to their target regions: the dorsal noradrenergic bundle, which contains most of the fibers from the LC; the median forebrain bundle, which contains fibers from the LC and the remaining nuclei; the dorsal longitudinal fascicles in the periaqueductal gray; and the medullary catecholamine bundle, which passes from the central tegmental tract to the caudal medulla (Stanford, 1995). Noradrenergic terminals in the hippocampal formation, the neocortex, and the olfactory bulb all originate in the LC. The LC is also the major source of neuronal fibers innervating the basal forebrain, thalamus, cerebellum, and spinal cord. No brain area is exclusively innervated by neurons from the medulla, although most fibers innervating the hypothalamus and the brain stem originate in the medulla.

The electrical discharge activity (''firing'') of LC neurons is accompanied by NA release (Aston-Jones *et al.,* 1984). In rats and monkeys, the pattern of spontaneous discharges of LC neurons, and thus release of NA, covaries with stages of the sleep–wake cycle: the neurons fire fast during waking, more slowly during slow-wave sleep, and become virtually silent during paradoxical sleep (Foote and Aston-Jones, 1995). A strong relationship exists among the tonic LC discharge rate, sensory responsiveness of LC neurons, and vigilance performance. Thus, periods of drowsiness are accompanied by decreased LC discharge, whereas alertness is consistently associated with high LC activity. Discharge rates are not only reduced during periods of low arousal (drowsiness or sleep), but also during certain behaviors (grooming and feeding), during which the animal is an active waking state but inattentive to most environmental stimuli (Aston-Jones *et al.,* 1991; Foote and Aston-Jones, 1995). In rats and monkeys, the highest discharge rates observed were associated with spontaneous or evoked behavioral orienting responses. LC discharge associated with orienting behavior is phasically most intensive when automatic, tonic behaviors such as sleep, grooming, or food consumption are suddenly interrupted, and the animal orients toward the external environment. Optimal focusing of attention occurs with intermediate levels of tonic LC activity. In the cerebral cortex, NA or LC stimulation ''gates'' inputs to target neurons so that

subthreshold synaptic inputs can become suprathreshold and elicit electrical discharge activity in the target neuron (Waterhouse *et al.,* 1988; Mouradian *et al.,* 1991). Evidence shows that NA can enhance both excitatory and inhibitory inputs to neocortical target cells. In the visual cortex of rats, NA application was found to enhance the vigor and the precision of visually evoked responses. In the hippocampal formation, exogenous NA administration or LC stimulation has been shown to increase the amplitude of the population spike evoked by perforant path stimulation of the dentate gyrus (Foote and Aston-Jones, 1995).

Early studies by Aghajanian and colleagues provided evidence for the inhibition of noradrenergic LC neurons by α_2-adrenergic agonists such as clonidine (Foote *et al.,* 1983). *In vivo* data indicated that one possible role of α_2-adrenoceptors is autoinhibition of the neurons (Aston-Jones *et al.,* 1990). It is now known that stimulation of these receptors located on the collaterals of LC neurons (fibers that project onto fibers of the same neuron) inhibits firing of these neurons and at the same time inhibits further release of NA. In accord with this view, electrophysiological studies show that NA and adrenaline strongly suppress LC impulse activity. α_2-ARs are the most prominent adrenoceptors in this nucleus and are located on LC neuron collaterals (Lee *et al.,* 1998a).

Stress leads to an activation of monoaminergic systems in the brain and there is a strong link between stress and the activation of the LC system (Thierry *et al.,* 1968; Valentino and Aston-Jones, 1995). The earliest studies on this issue demonstrated that stress was associated with increased NA turnover in brain regions known to receive their sole NA input from the LC (e.g., hippocampus and cortex; Valentino *et al.,* 1993). In animals, physiological challenges such as hypoglycemia, heat, hypotension, restraint, and aversive auditory stimuli increase LC discharge, a mechanism that also activates the autonomic nervous system (Valentino *et al.,* 1993). However, coactivation of sympathetic and LC activity does not always occur, and the two effects are not always temporally correlated, suggesting that sympathetic stimulation is neither the initial stimulus for nor a consequence of LC activation. A likely cause of enhanced NA release during stress is increased discharge of LC neurons, which is supported by findings that stress increases the expression of tyrosine hydroxylase, the rate-limiting enzyme of the noradrenaline biosynthesis pathway in LC cell bodies. Various stressors such as hypotension, distension of the urinary bladder, nicotine, opiate withdrawal, or foot shock activate LC neurons via different neurotransmitter inputs, e.g., involving the ''stress peptide'' corticotropin-releasing factor and the excitatory transmitter glutamate (Valentino and Aston-Jones, 1995). Via such mechanisms, stress may influence cognitive functions as noradrenergic projections from the LC to the cortex modulate cortical sensory input (Robbins and Everitt, 1995). Besides the function in

the regulation of vigilance and attention, many other roles of this transmitter have also been described (Aston-Jones *et al.,* 1990; Fillenz, 1990).

Adrenaline-producing neurons are only present in low numbers in the brain. There are two groups of adrenergic neurons, which are both located in the medulla oblongata: C1 and C2 (Hökfelt *et al.,* 1984). A third group, C3, located in the vicinity of the hypoglossal nucleus, has been described only for the rat. C1 and C2 adrenergic neurons project to the hypothalamus; C1 neurons also project to the spinal cord. Functions of adrenaline partly overlap with those of noradrenaline because both these catecholamines can stimulate ''adrenergic'' receptors, although with different affinity (see later). However, stress-induced changes in brain adrenaline and PNMT activity indicated a specific role of this transmitter in hypertension and blood pressure regulation (Saavedra, 1988).

B. Serotonin

Serotonin is synthesized from tryptophan, an essential amino acid that is preferentially taken up by serotonergic neurons through catalysis of the enzyme tryptophan hydroxylase (Azmitia and Whitaker-Azmitia, 1995). The serotonin system in the mammalian brain is a very expansive and complex anatomical/neurochemical entity that has been reviewed in great detail (Jacobs and Azmitia, 1992). Neurons that produce serotonin are restricted to nuclei in the brain stem that innervate nearly every area of the brain. Brain stem serotonergic nuclei are located on and near the midline and can be divided into the superior and the inferior group. The superior group consists of four nuclei that innervate the forebrain: caudal linear nucleus (group B8), median raphe nucleus (also called nucleus centralis superior; groups B8 and B5), the B9 group in the nucleus pontis oralis, and the dorsal raphe nucleus (B6 and B7). The nuclei of the inferior group (nucleus raphe obscurus, B2; nucleus raphe pallidus, B1 and B4; nucleus raphe magnus, B3; neurons in the ventrolateral medulla, and the intermediate reticular nuclei, B1/B3) innervate the spinal cord, thus forming the descending system. Serotonergic neurons and their fibers have a high capacity to regenerate. Even in the adult brain, damaged serotonergic fibers can survive an injury and, with a certain latency, reinnervate the denervated regions.

Serotonergic neurons in the raphe nuclei are autoactive and discharge in a stereotyped almost clock-like manner. Electrical recordings showed a slow and highly regular discharge pattern with one to two spikes/second, which led to the hypothesis that the neurons might be driven by an intrinsic pacemaker mechanism (Aghajanian *et al.,* 1968; Aghajanian and Vandermaelen, 1982a). Double-labeling techniques were applied to identify these

neurons as really serotonin-producing cells (Aghajanian and Vandermaelen, 1982b). Serotonergic neurons of the dorsal raphe nucleus can be inhibited by their own transmitter, 5HT, which controls their rhythmic activity via a negative feedback mechanism. When the level of extracellular 5HT increases, e.g., following administration of the precursor L-tryptophan, the activity of the serotonergic neurons decreases (Aghajanian, 1972). This is due to stimulation of $5HT_{1A}$-autoreceptors located on the soma and proximal dendrites of the serotonergic neurons (see later). It is presumed that the decrease in 5HT release induced by the $5HT_{1A}$-autoreceptors is a homeostatic response that compensates for high synaptic levels of the transmitter.

The discharge pattern of neurons in the dorsal raphe nucleus and the release of 5HT changes across the sleep–wake–arousal cycle (Trulson and Jacobs, 1979). Studies in cats showed that the stable pattern of slow and regular discharge activity occurs only when the animal is in a state of undisturbed waking (''quiet waking''). As the animal becomes drowsy and enters slow-wave sleep, neuronal activity decreases, meaning that the discharge rate slows down and loses its regularity. During sleep, this culminates, and during rapid eye movement sleep, serotonergic neurons are completely inactive. Reciprocal to the low activity during sleep is a high activity when the animal becomes behaviorally active. An aroused state goes together with an approximately 40% increase in the activity of 5HT neurons.

In coincidence with the wide expansion of the serotonergic structures in the brain, the system has been suggested to be involved in almost every function the brain could subserve: regulation of cardiovascular and respiratory activity, motor output, neuroendocrine secretion, sleep, nociception, analgesia, aggression, mood, and last but not least depression (Whitaker-Azmitia and Peroutka, 1990). According to Jacobs and Fornal (1995), the primary function of 5HT is to facilitate gross motor output. The slow and rhythmic regular firing of 5HT neurons during quiet waking creates a steady-state release of the transmitter that provides a tonic excitatory drive for the activity of motor neurons. During gross repetitive motor behaviors mediated by the brain stem and spinal cord, subpopulations of 5HT neurons are activated, attaining discharge levels several times greater than those observed during undisturbed waking. In contrast, when the 5HT system is inactivated, tonic motor output is disfacilitated and, at the same time, sensory information processing is now facilitated.

Although many reports implicate that serotonergic neurons in the brain control physiological processes such as stress reactions, there are no indications that the raphe neurons change their discharge rate in acute stress (Jacobs and Azmitia, 1992). However, as described later, chronic stress has an impact on serotonin receptors in target regions of the serotonergic raphe neurons.

III. Monoamine Receptors

Monoamine receptors belong to the superfamily of G-protein-coupled receptors, which are integral membrane proteins characterized by amino acid sequences containing seven hydrophobic domains that constitute the transmembrane-spanning regions of the receptor molecule (Watson and Arkinstall, 1994; see Fig. 1). Being located in the plasma membrane of many cells, they are involved in the transmission of signals from the exterior to the interior of a cell through interaction with heterotrimeric G-proteins that are situated inside the plasma membrane (Fig. 2). The G-protein complex, which consists of α, β, and γ subunits, serves as a transfer machinery that transduces a signal to various effectors at the intracellular side of the plasma membrane. The α subunit contains a guanine nucleotide-binding site and interacts with the receptor. On receptor activation, the G-protein complex dissociates into the subunits Gα (that binds the GTP) and G$\beta\gamma$, which leads to stimulation of a cascade of intracellular events involving effector systems such as adenylate cyclase, receptor kinases, phospholipases, phosphodiesterases, and ion channels (Gilman, 1987; Watson and Arkinstall, 1994; Strader *et al.,* 1995; Clapman, 1996; Schneider *et al.,* 1997).

Monoamine receptors such as adrenergic and serotonergic receptors are encoded by single exons. The binding site for agonists (adrenaline, nor-

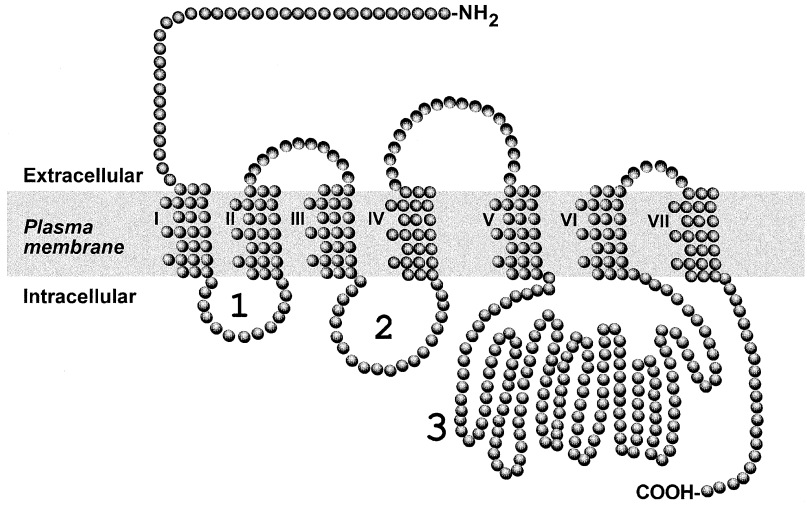

FIG. 1 Molecular structure of the human α_{2A}-adrenoceptor (according to McDonald *et al.,* 1997). Circles represent the amino acids, roman numbers indicate the seven hydrophobic transmembrane domains, and latin numbers denote intracellular loops. The large third intracellular loop is characteristic for α_2-adrenoceptors.

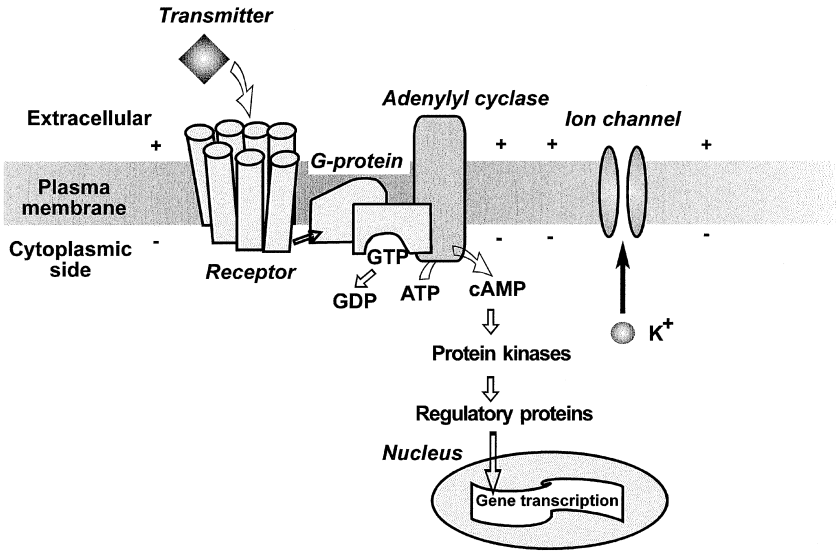

FIG. 2 Signal transduction by G-proteins. G-protein-coupled receptors are membrane-bound proteins characterized by seven transmembrane-spanning regions. The G-protein complex, which consists of α, β, and γ subunits, serves as a transfer machinery that transduces a signal to various effectors at the intracellular side of the plasma membrane. The α subunit contains a guanine nucleotide (GTP)-binding site and interacts with the receptor. Upon receptor activation by the transmitter, the G-protein complex dissociates into the subunits Gα and G$\beta\gamma$. This leads to stimulation of a cascade of intracellular events involving effector systems such as adenylate cyclase, receptor kinases, phospholipases, phosphodiesterases, and ion channels (Gilman, 1987; Watson and Arkinstall, 1994; Clapman, 1996; Schneider *et al.,* 1997).

adrenaline, serotonin, or their analogs), which activate the receptors, is contained in the transmembrane regions, which form a pocket to take up the receptor ligand. Although the sequences of the transmembrane regions are highly conserved among members of the receptor family, a greater divergence in amino acid sequences is found in the extracellular regions, which thus contribute to receptor subtype specificity. Binding mechanisms of agonists have been well studied in adrenoceptors (Gether *et al.,* 1995). The carboxylate group on the conserved aspartate residue in the third transmembrane domain probably acts as a counterion for the nitrogen in the catecholamine molecule, whereas two serines in the fifth transmembrane domain interact with the hydroxyl groups on the catechol ring (Strader *et al.,* 1994). Binding sites for antagonists (ligands that block the receptor) are often distinct from those of naturally occurring ligands.

Monoamine receptors can have a higher molecular weight than predicted from their amino acid sequence, suggesting that they are glycosylated.

Glycosylation occurs in a consensus sequence in the extracellular domain, which contains one or more asparagine residues (Strader *et al.,* 1994). Direct evidence for glycosylation has been found for a number of receptors, including β_2-adrenoceptors in which endoglycosidase treatment decreases the molecular mass from approximately 65,000 to 49,000 Da (Benovic *et al.,* 1987; Rands *et al.,* 1990). For most receptors it is believed that glycosylation plays only a minor role in agonist binding but it may be of importance in determining the correct distribution of receptor molecules within the cell. Two cysteine residues, one in the first and one in the second extracellular loop, which are present in all G-protein-coupled receptors, are essential for stabilizing the tertiary structure of the receptor. In β_2-ARs, these cysteine residues are also important for the interaction of the receptor with agonists and antagonists (Dohlman *et al.,* 1987). Other cysteine residues can be substrates for palmitoylation. The whole three-dimensional structure of the receptor molecule at the cytoplasmic side appears to be important for the interaction with the G-protein. The β_2-AR has a cysteine residue in its C-terminal sequence that binds palmitate via a thioester linkage (O'Dowd *et al.,* 1989). It was suggested that this covalently bound palmitic acid residue becomes intercalated into the lipid bilayer of cellular membranes.

Responses of G-protein-coupled receptors undergo rapid desensitization during continued exposure to agonists and there is evidence that protein phosphorylation is involved in these events. The intracellular portions of G-protein-coupled receptors are rich in serine and threonine residues, which can be phosphorylated, and many of these residues are found in consensus sequences for phosphorylation by protein kinases such as protein kinase A (PKA) and protein kinase C (PKC). Studies on β_2-ARs have shown that binding of an agonist induces a conformational change in the receptor molecule, which activates β-AR kinase (BARK) to phosphorylate serine and threonine residues in the intracellular carboxyl-terminal tail (Dohlman *et al.,* 1987; Bouvier *et al.,* 1988).

A. Adrenergic Receptors

Adrenergic receptors are divided into three main types based on nucleotide sequence, receptor pharmacology, and intracellular signaling mechanisms. Further subdivisions exist within each class. A large number of agonists and antagonist can, to a certain extent, distinguish among the three main classes of adrenoceptors: α_1, α_2, β_1, β_2, and β_3-ARs. The following paragraphs summarize current knowledge on α_2 and β-ARs. A description of α_1-ARs is beyond the scope of this review (Watson and Arkinstall 1994; Bylund *et al.,* 1994).

1. α_2-Adrenoceptors

a. Molecular Biology Three α_2-adrenoceptor subtypes (A, B, and C) are now known that share many common properties. A fourth subtype, D, mentioned in some publications on rat and bovine tissues, is considered as a species ortholog of subtype A (O'Rourke *et al.,* 1994b). The three members of the α_2-AR family are all known to inhibit adenylate cyclase through coupling to an inhibitory G-protein (G_i), although some members of the family can also couple to a stimulatory G-protein (G_s) under some circumstances (Rohrer and Kobilka, 1998). The subtypes are encoded by distinct genes, localized on different chromosomes, and are well conserved among mammalian species (MacDonald *et al.,* 1997). The genes for the human α_2-ARs have been sequenced and their localization on the different chromosomes was determined. The gene for subtype A is located on chromosome 10 (Kobilka *et al.,* 1987), the gene for subtype B on chromosome 2 (Regan *et al.,* 1988), and the gene for subtype C on chromosome 4 (Lomasney *et al.,* 1990). Sequences of rat α_2-ARs (Zeng *et al.,* 1990; Flordellis *et al.,* 1991; MacKinnon *et al.,* 1994), all three guinea pig α_2-ARs (Svensson *et al.,* 1996), porcine α_{2A}-AR (Guyer *et al.,* 1990), and oppossum α_{2C}-AR (Blaxall *et al.,* 1994) are known. Furthermore, partial sequences of the bovine and chicken α_{2A}-ARs have been published (Blaxall *et al.,* 1993; MacDonald *et al.,* 1997). Subtypes A, B, and C have been reported to be coupled to the $G_{i/o}$ signaling system, to inhibit the activity of adenylate cyclase, to inhibit the opening of voltage-gated Ca^{2+} channels, and to open K^+ channels (Boehm *et al.,* 1996).

b. Regulation of α_2-AR Subtypes Depending on their cellular localization, α_2-ARs display different physiological functions and pharmacological activity profiles. Due to specific regulatory regions in the receptor genes and protein structures (e.g., consensus sites for kinase-mediated phosphorylation reactions) the properties of the three subtypes differ with regard to receptor synthesis and posttranslational events. A common phenomenon with G-protein-coupled receptors is ''desensitization,'' which means that the biological response is dampening when the receptor is exposed continuously to a stimulus (e.g., to the agonist). Three processes have been shown to be associated with adrenoceptor desensitization: (i) a rapid uncoupling of the receptor from effector units, (ii) rapid sequestration of the receptor away from the cell surface to intracellular compartments, and (iii) downregulation, which takes place over several hours and results in a decrease in receptor number (Collins *et al.,* 1990; for more details see Section III,A,2). To investigate desensitization mechanisms of α_2-ARs, genes for the three subtypes (clones α_2 C10, $C2_1$, and C4 representing subtype A, B, and C, respectively) were transfected into specific cell lines, and receptor coupling to G-proteins and regulation was analyzed. It was found that the subtypes

differ in terms of G-protein coupling and short-term agonist-promoted desensitization by phosphorylation (Eason and Liggett, 1992), long-term downregulation (Eason and Liggett, 1992; Eason *et al.,* 1994), and agonist-promoted intracellular sequestration (Eason and Liggett, 1992). Subtype A undergoes short-term agonist-induced desensitization via receptor phosphorylation, probably mediated by a G-protein-coupled receptor kinase, and subtype C can also undergo short-term functional desensitization accompanied by phosphorylation, but subtype B is not phosphorylated. In these processes, the activation of kinases also occurs in a receptor subtype-specific manner (Liggett, 1997). Furthermore, the α_{2A} and the 2C-AR can be glycosylated, and α_{2A} and α_{2B} receptor molecules can be acylated (Kennedy and Limbird, 1994; MacDonald *et al.,* 1997). Studies with cell lines showed that when stimulated by agonists, α_2-ARs can be internalized into the cell following the same endosomal pathway as β_2-ARs (see later). This process is also receptor specific, and experiments with cell lines expressing the different α_2-ARs suggested that subtypes B and C are internalized, whereas subtype A is not (Daunt *et al.,* 1997). Regulation of receptor gene expression has been investigated with subtype C and it was shown that the 3′-untranslated region of the α_{2C} mRNA determines the efficacy of receptor protein synthesis (Yang *et al.,* 1997).

c. Distribution of α_2-ARs in the Brain The neuroanatomical distribution of monoamine receptors can be studied by *in vitro* receptor autoradiography (Fig. 3). Detailed mappings of α_2-ARs have been performed by receptor autoradiography in adult brains of several species such as humans (Zilles *et al.,* 1993), rats (Young and Kuhar, 1980; Boyajian *et al.,* 1987; Unnerstall and Kuhar, 1988; Herbert and Flügge, 1995), tree shrews (Flügge *et al.,* 1990; Flügge *et al.,* 1993a,b), mice (Sallinen *et al.,* 1997), chickens (Dermon and Kouvelas, 1989), and Japanese quail (Ball *et al.,* 1989). The receptors are present in many regions of the forebrain (cortex, limbic regions, hypothalamus), the hindbrain (pons, medulla), and in the spinal cord (Flügge *et al.,* 1992; see Fig. 4). Pharmacological studies also demonstrated the presence of α_2-ARs in the cortex of the mouse (Limberger *et al.,* 1995). The expression of subtype-specific α_2-AR mRNA in distinct brain areas was visualized by *in situ* hybridization using cRNA or oligonucleotide probes (McCune *et al.,* 1993; Nicholas *et al.,* 1993a; Winzer-Serhan *et al.,* 1997a,b). Messenger RNA coding for all three α_2-ARs was detected in the central nervous system (CNS), revealing a widespread distribution of subtypes A and C, but mRNA for subtype B is found primarily in the thalamus (Scheinin *et al.,* 1994). High expression of α_{2A}-AR mRNA was found in the LC and in areas that are involved in cardiovascular control (such as in brain stem and hypothalamus), in limbic structures (such as amygdala), in the whole cerebral cortex, and in the spinal cord. α_{2C}-AR mRNA is more abundant

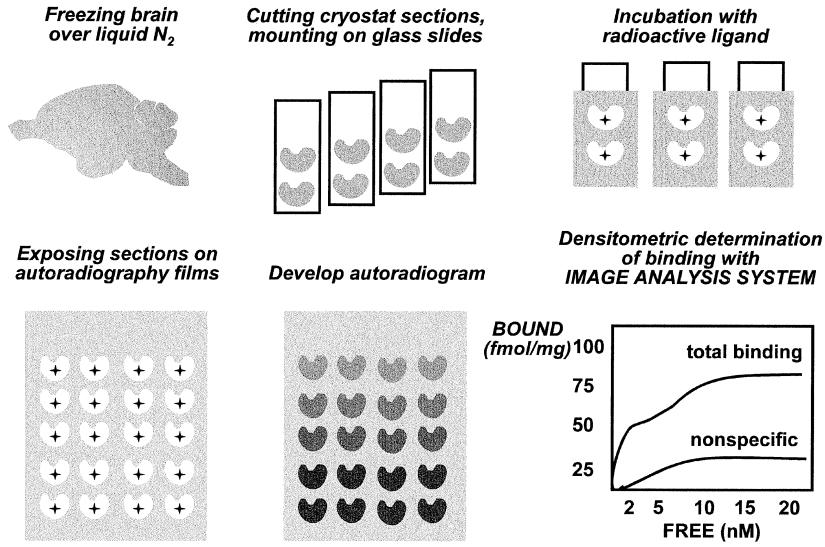

FIG. 3 *In vitro* receptor autoradiography: Brains are dissected quickly postmortem and frozen over liquid nitrogen. Thin sections (10 μm) are cut in a cryostat, mounted on glass slides, and incubated with receptor-specific radioactive ligands dissolved in buffer solutions. Radioactive sections are then exposed on autoradiography films and, after appropriate exposure time, autoradiograms are developed. Binding of the radioactive ligand is analyzed densitometrically using an image analysis system (according to Kuhar and Unnerstall, 1985). Total and nonspecific binding of the radioligand is determined for the evaluation of specific binding. The maximal specific binding of the radioligand (B_{max} value) gives the number of receptors, whereas the steepness of the binding curve is related to the receptor affinity (expressed as K_d value).

in the basal ganglia, olfactory regions, hippocampal formation, and cerebral cortex.

After the production of subtype-specific antibodies it was possible to track down the receptors precisely to discrete neuroanatomical structures (Talley *et al.,* 1996; Rosin *et al.,* 1996). These immunocytochemical studies basically confirmed the mapping studies performed with *in vitro* receptor autoradiography and, in addition, allowed to localize the receptors on the subcellular level. Thus, electron microscopic experiments demonstrated that α_{2A}-ARs in the locus coeruleus are associated with the plasma membrane of dendrites of tyrosine hydroxylase immunopositive neurons (representing the noradrenergic LC neurons) and are found at axon terminals and in processes of glial cells (Lee *et al.,* 1998a). Subtype C is also present on dendrites of tyrosine hydroxylase immunopositive LC neurons (Lee *et al.,* 1998b). In the hippocampus, α_{2A}-ARs were visualized on catecholamin-

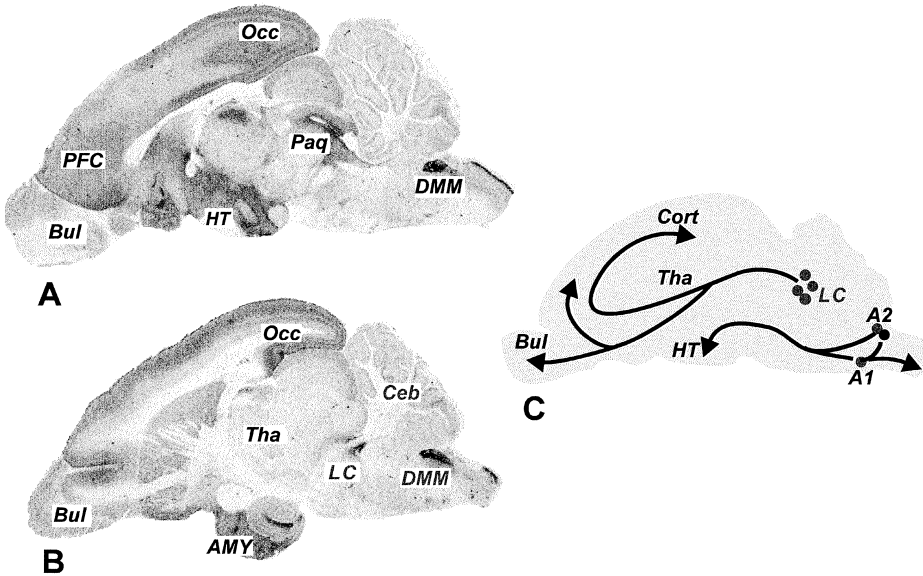

FIG. 4 α_2-Adrenoceptors in the tree shrew brain visualized by receptor autoradiography with the radioligand [^{3}H]rauwolscine (A, B). Autoradiograms show sagittal sections from the anatomical level close to the midline (A) and 3 mm lateral from the midline (B). Black areas demonstrate the presence of high numbers of receptors and white areas show no receptors. Note the high number of receptors in the locus coeruleus (LC) and the dorsomedial medulla oblongata (DMM). (C) The main noradrenergic centers (LC in the pons, A2 in the dorsomedial medulla oblongata, A1 in the vertrolateral medulla) and their projections to the forebrain (left) and the spinal cord (right). AMY, amygdala; Bul, olfactory bulb; Ceb, cerebellum; Cort, cerebral cortex; Occ, occipital cortex; Paq, periaqueductal gray; PFC, prefrontal cortex; HT, hypothalamus; Tha, thalamus.

ergic dendrites belonging to fibers innervating the hippocampus and on dendritic spines of pyramidal and granule cells (Milner *et al.,* 1998). In the lower brain stem, catecholaminergic and serotonergic neurons innervating the spinal cord were immunopositive for α_{2A}-ARs (Guyenet *et al.,* 1994). In the visual cortex of rats, pyramidal and nonpyramidal neurons carry α_{2A}-ARs and, during development, receptors are already present prior to synaptogenesis (Venkatesan *et al.,* 1996). In the primate fetus, α_{2A}-ARs are expressed by diverse cell types in the proliferating zones of the embryonic cerebral wall (Wang and Lidow, 1997). In the adult monkey prefrontal cortex, the majority of α_{2A}-ARs are located in the preterminal regions of axons in opposition to noradrenergic fibers (Aoki *et al.,* 1998). In the rat spinal cord, immunoreaction products for subtypes A and C were localized on different neuronal structures (Stone *et al.,* 1998). The primary localiza-

tion of subtype A receptors in the spinal cord is on terminals of substance P containing fibers (the primary afferent nociceptive fibers), suggesting that these are sites for the analgesic actions of α_2-adrenergic agonists.

d. Pharmacology of α_2-ARs Originally, α_2-ARs have been distinguished from α_1-ARs by their low affinity for prazosin (MacKinnon *et al.,* 1994). To date, there are several drugs that bind specifically to α_2-ARs, but compounds that bind to only one receptor are still not available. In addition, α_2-AR ligands that are imidazoles (dexmedetomidine, atipamezole) or imidazolines (clonidine, idazoxan) also bind with moderate to high affinity to nonadrenoceptor imidazol(in)e binding sites (French, 1995; Wikberg *et al.,* 1991). The α_2-AR selective antagonist RX821002 has a high affinity for all three subtypes (O'Rourke *et al.,* 1994a; Halme *et al.,* 1995). However, some ligands show a certain degree of subtype selectivity. Thus, oxymetazoline and prazosin are widely used to distinguish α_{2A}-ARs from the other subtypes (MacKinnon *et al.,* 1994), and the antagonists rauwolscine and yohimbine have been reported to label preferentially the rat α_{2C} subtype (Marjamäki *et al.,* 1993). The antagonist MK912 showed an approximately 10-fold higher affinity for subtype C than for A and B (Uhlen *et al.,* 1994). Finally, the agonist UK14,304 has at least 10 times higher affinity for α_{2A} than for α_{2C} and can therefore be used to distinguish between the two subtypes.

e. Role of α_2-ARs in Physiology and Pathophysiology α_2-ARs function as autoreceptors that regulate the release of neuronal noradrenaline (Fig. 5). On the basis of pharmacological experiments with peripheral sympathetic nerves, it was proposed that they are located on noradrenergic terminals where they regulate the release of noradrenaline via a negative feedback mechanism (Starke, 1987). Similar processes take place in the brain where α_2-AR stimulation inhibits the release of noradrenaline from noradrenergic neurons (Fillenz, 1990). The supposition that the receptors are located on processes of noradrenergic cells was confirmed by the previous-mentioned morphological studies with receptor specific antibodies demonstrating α_2-AR subtype A immunoreactive products in cells which also contain tyrosine hydroxylase (Lee *et al.,* 1998a). However, there are also nonadrenergic structures, e.g., glial cells, that carry α_2-ARs. On the basis of pharmacological experiments, it was proposed that such "heteroreceptors" exist on serotonergic neuronal fibers (Arbilla and Langer, 1990).

Important membrane processes initiated by α_2-AR stimulation are the blockade of N-type voltage-gated Ca^{2+} channels detected by electrophysiological methods in several neurons (Boehm *et al.,* 1994; Diversé-Pierluissi *et al.,* 1996) and the increase in potassium conductance, e.g., found in LC neurons (Williams *et al.,* 1985). This stimulation of potassium currents, which is due to the activation of inwardly rectifying G_i-protein-gated K^+

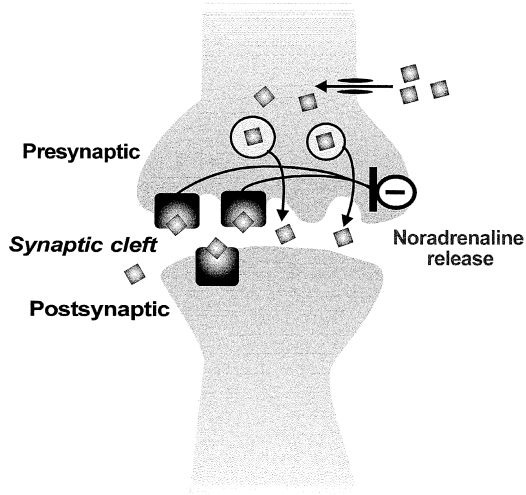

FIG. 5 Feedback inhibition of noradrenaline release: noradrenaline (squares) released from the nerve terminal binds to presynaptic α_2-adrenergic autoreceptors. This blocks further release of the transmitter (according to Starke, 1987). In addition to presynaptic α_2-adrenoceptors, there are also postsynaptic α_2-adrenoceptors, which modulate the activity of cells that do not produce noradrenaline.

channels, causes neuronal hyperpolarization and, concomitantly, a decrease in the firing rate of excitable cells in the central nervous system, which is probably the physiological basis for the analgesic and sedative effects of α_2-AR agonists (Birnbaumer *et al.,* 1990; Aantaa and Scheinin, 1993). Thus, the agonist clonidine acts as a sedative and induces sleep, decreases exploratory behavior, and suppresses the startle response in the rat (Aantaa and Scheinin, 1993). The hypnotic and anesthetic actions of the agonist dexmedetomidine are known to involve coupling to a G_i-protein and increased K^+ conductances (Doze *et al.,* 1990). An important site for the analgesic actions appears to be the LC because dexmedetomidine injection into this nucleus produced antinociception (Guo *et al.,* 1996). An intracellular process induced by α_2-AR stimulation is the reduction in 3′,5′-cyclic adenosine monophosphate (cAMP), and this second messenger itself is an important regulator of many cellular functions (Limbird, 1988). The changes in intracellular Ca^{2+} concentrations, which are induced by the inhibition of calcium influx, can also have a strong impact on many intracellular events.

It has been known for many years that central nervous α_2-ARs play a part in the mechanisms that regulate blood pressure. Hypertensive rats show different α_2-AR-binding characteristics than normotensive rats, and it was reported that catecholamines influence the baroreceptor reflex via

α_2-ARs (Kubo *et al.,* 1990). A high density of α_2-ARs has been observed in the dorsal motor nucleus of the vagus, the nucleus in the brain stem that receives afferents from the vagal nerve and innervates visceral organs such as the stomach, indicating that the central nervous control of visceral organ functions involves α_2-ARs (Loewy and Spyer, 1990). The dorsal motor nucleus of the vagus may be the site for bradycardic and hypotensive effects of α_2-AR activation (Unnerstall *et al.,* 1984). The hypothesis that central nervous hypotensive effects of noradrenaline are mediated by α_2-ARs was verified by gene-engineering experiments: mice with a point mutation in the gene for subtype A, which substitutes the aspartate residue at position 79 (in the second transmembrane domain) by an asparagine (D79N mutation), do not show the hypotensive responses to α_{2A}-AR agonists that wild-type mice display (MacMillan *et al.,* 1996). Functional changes in the mutant are accompanied by failure to inhibit voltage-gated Ca^{2+} currents and spontaneous neuronal firing, and there are indications for failure of K^+ current activation (Lakhlani *et al.,* 1997). It appears that due to the D79N mutation, the receptor molecule cannot achieve the conformation necessary to activate the G-protein (Surprenant *et al.,* 1992). In addition to the loss of the wild-type membrane effects and the deficit in α_{2A}-AR-mediated hypotensive effects, the D79N mutation also abolished the antiepileptogenic actions of noradrenaline (Janumpalli *et al.,* 1998). In addition to the central nervous receptor, peripheral α_2-ARs are of course also involved in the regulation of cardiovascular function. Stimulation of the receptors in arterial smooth muscle cells raises blood pressure by increasing vascular resistance (Parkinson, 1990). The arterial blood pressure response to dexmedetomidine in wild-type mice is biphasic with an immediate hypertensive response followed by a long-lasting drop in mean arterial pressure (for more than 1 h). In genetically engineered mice (knockouts) with a deficit in α_2-AR subtype B, there was no initial hypertensive phase after dexmedetomidine injection but an immediate strong hypotensive response, indicating that in the periphery, it is subtype B that has a strong impact on vascular tone (Link *et al.,* 1996).

Because of the widespread distribution of α_2-ARs in the brain, these receptors are not only involved in blood pressure regulation and analgesia, but also affect other functions. Thus, peripheral injections of dexmedetomidine not only induce hypotension, bradycardia, and sedation, but also hypothermia, inhibition of locomotor activity, and anxiolysis. A mouse strain with an inactivated α_{2c}- gene showed an attenuation of the agonist-mediated hypothermic response, whereas another strain with a threefold overexpression of α_{2c}-ARs showed an accentuated hypothermic response (Sallinen *et al.,* 1997). The hypothermic effect of the agonist thus appears to be mediated, at least in part, by subtype C.

α_2-ARs appear to be involved in the regulation of behavior, cognitive function, and mood. Pharmacological agents that affect LC firing modulate behaviors (Bremner *et al.,* 1996a). Administration of the antagonist yohimbine, which results in increased LC firing, induced behavioral reactions in rats and monkeys that reflect anxiety or fear (Redmond and Leonard, 1997). In rats, certain α_2-AR blockers also modified coping strategies, inducing a shift from a passive to a more active coping style (Haller *et al.,* 1995). Modulation of cognitive function was shown in young hyperactive, inattentive monkeys, where a single subsedative dose of the agonist clonidine had a calming effect, which in the cognitive test situation (a delayed response task) resulted in improved performance on the task (Arnsten *et al.,* 1996). The just-mentioned mice with the targeted inactivation of the α_{2c}-AR gene showed enhanced startle responses, i.e., a shortened reaction time to a strong acoustic stimulus, a behavior showing some parallels to fear reactions in humans. In addition, they displayed shortened attack latencies in a test for aggressive behavior (Sallinen *et al.,* 1998). Furthermore, α_2-AR changes in the brain have been proposed to be involved in the etiology of depression. In postmortem material from the prefrontal cortex of suicide victims, a low-affinity [^{3}H]clonidine-binding site was missing that was present in healthy subjects (Andorn, 1991). In another study, the binding capacity of the agonist ligand [^{3}H]UK14,304 was found to be increased in the frontal cortex of suicide victims, and findings performed with the antagonist ligand [^{3}H]RX821002 on the neocortex of depressed patients revealed that a greater proportion of α_{2A}-ARs was in the high-affinity conformation for agonists compared to controls (Meana *et al.,* 1992; Callado *et al.,* 1998). Although these reports indicate a role of α_2-ARs in the central nervous processes of depression, the underlying mechanisms are still unclear. Regulation of α_2-AR gene transcription apparently does not play a major role, as a genetic study on subjects of a Japanese population showed no correlation between a polymorphism in the promotor region of that gene and the occurrence of mood disorders (Ohara *et al.,* 1998). However, as pointed out earlier, regulation of receptor numbers and function can take place on many levels of the intracellular machinery (e.g., mRNA processing, posttranslational receptor modification, proteolysis of receptor molecules).

2. β-Adrenoceptors

a. Molecular Biology and Regulation Three subtypes of β-adrenoceptors are now known, which are all coupled to G-proteins (Watson and Arkinstall, 1994). β_1- and β_2-ARs were originally distinguished according to their potency to bind the endogenous ligand adrenaline, which has a higher affinity for β_2-AR compared to β_1-AR. The β_3-AR has a low affinity for β-antagonists.

β-ARs are encoded by intronless genes. Human β_1-ARs consist of 477 amino acids (molecular mass approximately 51,000 Da) with a glycosylation site at the asparagine residue at position 15, a disulfide bond between cysteines at positions 131 and 209, and a palmitoylation site on the cysteine in position 392. The human β_1-AR gene is located on chromosome 10 (Yang-Feng *et al.,* 1990). The sequences of β_1-ARs from other mammalian species such as mouse are also known (Jasper *et al.,* 1993; Watson and Arkinstall, 1994). The human β_2-AR, encoded on chromosome 5, consists of 413 amino acids (molecular mass approximately 46,000 Da), with glycosylation sites at asparagines 6 an 15, a disulfide bond between cysteines at positions 106 and 184, and a palmitoylation site at the cysteine in position 341 (Dohlmann *et al.,* 1987). Stimulation of both β_1- and β_2-AR activates adenylate cyclase through interaction with G_s. The overall amino acid identity between human β_1 and human β_2-ARs is only 54%. Rat and mouse β_1-ARs show 98% overall identity in amino acid sequence, and 92% identity with the human β_1-AR. Species-related differences between rodent and human receptors are concentrated in the third cytoplasmic loop (Hieble and Ruffolo, 1995). The existence of a β_3-AR has been confirmed by receptor cloning. This ''atypical'' β-AR has a very limited distribution and is present in low levels in adipose tissue and the gastrointestinal tract where it stimulates lipolysis and increases gut motility.

It was shown in 1979 that β_1- and β_2-ARs are regulated independently (Minneman *et al.,* 1979). In the past years, the β_2-AR has been investigated in great detail to elucidate the relationship between the molecular structure and the regulation of adrenergic receptors (Liggett, 1995). Applying gene-engineering techniques (site-directed mutagenesis), it was found that the ligand-binding site is within the transmembrane-spanning sequences (TMS). Over the years, a concept emerged that the seven TMS are arranged in a ring within the cell surface membrane, forming a binding ''pocket'' for the ligand. The third intracellular loop is obviously the principal site for G_s coupling. This loop is connected to the fifth TMS domain where critical interactions with the agonist occur. Agonist binding induces a change in the conformation of the third intracellular loop, whereby its affinity for the G_s-protein is increased so that coupling between receptor and G-protein can occur. This coupling of the third intracellular loop to G_s can be modulated by phosphorylation, which results in a reduced ability to activate G_s and thus to reduced sensitivity of the signaling system (desensitization).

Desensitization is defined as a dampening of a response despite the continuing presence of a stimulus (Sibley and Lefkowitz, 1985; Benovic *et al.,* 1986). It is thought to represent an adaptation to repetitive or intense stimuli and may contribute to dysfunction in pathological states. For the β_2-AR, three desensitization mechanisms have been delineated: phosphorylation, sequestration, and downregulation. (i) Desensitization by phosphor-

ylation occurs after brief exposure to an agonist and is characterized by rapid uncoupling of the receptor from G_s due to modification of the receptor molecule through the actions of kinases. These enzymes, e.g., cAMP-dependent PKA and BARK, are widely distributed at synapses in the brain (Arriza *et al.,* 1992). (ii) Sequestration takes place after longer agonist stimulation of β_2-ARs and means that receptor molecules are internalized from the cellular membrane into the cytoplasm and then into intracellular vesicles (Hausdorff *et al.,* 1990; Fig. 6). Sequestration is maximal after approximately 1 h of agonist exposure and it is cell type dependent (Liggett, 1995). It has been proposed that receptors are dephosphorylated and subsequently recycled to the cell surface during sequestration. Sequestration is not necessarily dependent on agonists occupying the receptor, but an increase in intracellular cAMP can also lead to reduced numbers of functional receptor. (iii) Downregulation takes place during prolonged agonist exposure (hours) and represents a decrease in receptor expression. This loss of functional β_2-ARs can be related to the loss of receptor-specific mRNA, which itself may be due to reduced transcription or to increased mRNA degradation. Thus, experiments with certain cell lines showed that glucocorticoids can downregulate β_1-adrenergic receptor expression by suppressing transcription of the receptor gene (Kiely *et al.,* 1994). However, expression of β_2-ARs was enhanced severalfold within hours of glucocorticoid adminis-

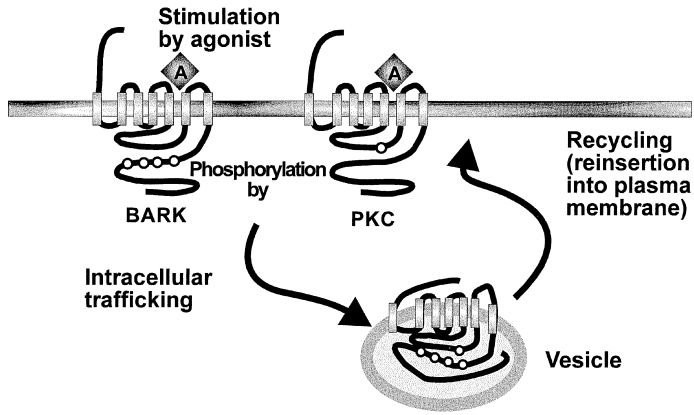

FIG. 6 Intracellular sequestration of β-adrenoceptors, which results in redistribution of the receptor molecules. After stimulation by an agonist (A), the receptor molecules in the membrane are phosphorylated by the β-adrenoceptor kinase (BARK) or protein kinase C (PKC). Phosphorylation sites are indicated by open circles. The receptor is then internalized to an intracellular compartment (probably endosomes). Sequestered receptor molecules can be recycled back to the plasma membrane as fully functional receptors (according to Liggett, 1995).

tration (Hadcock *et al.,* 1989). Elimination of a glucocorticoid response element in the 5′-untranslated region of the hamster β_2-AR gene abolished the transcriptional activation by the steroid (Malbon and Hadcock, 1988). However, nonsteroidal agents such as the morphogen retinoic acid can also regulate the expression of β-ARs (Tholanikunnel *et al.,* 1995). In addition, the proteolytic degradation of receptor molecules may also play a part in downregulation. β_2-ARs exist in multiple forms within the human population, and this polymorphism might be responsible for individual differences in receptor regulation. The most common polymorphism (transition of arginine to glycine at amino acid position 16) imparts an enhanced agonist-promoted downregulation of the receptor whereas the glutamine to glutamic acid transition at position 27 results in relative resistance to downregulation (Green *et al.,* 1994; Liggett, 1995).

b. Pharmacology and Distribution of β-ARs in the Brain Due to the role of β-ARs in cardiac and pulmonary physiology, the pharmacology of these receptors has been investigated extensively (Hieble and Ruffolo, 1995). All three β-AR subtypes can be activated by noradrenaline and adrenaline, but in contrast to α-ARs, β-ARs have different affinities for these catecholamines. β_1-ARs bind adrenaline and noradrenaline with almost equal affinity, whereas β_2-ARs bind adrenaline with higher affinity than noradrenaline. The synthetic catecholamine isoproterenol is a potent agonist at all three subtypes, and propranolol is an example of a nonselective antagonist. The affinity of the antagonist CGP 20712A for β_1 is higher than for β_2, whereas the antagonist ICI 118551 has a preference for β_2 compared to β_1 (Dooley *et al.,* 1986; Mauriege *et al.,* 1988). However, the receptor selectivity of β-adrenergic ligands is limited, and possible interactions with other classes of receptors have to be taken into consideration. Thus the radiolabeled antagonist ^{125}I-iodocyanopindolol, which has been used in many studies on the distribution of β-ARs in various tissues, also interacts with serotonergic receptors (Hoyer *et al.,* 1985). The distribution of β_1- and β_2-ARs in the brain has primarily been studied using iodinated derivatives of pindolol and it became clear from these studies that high levels of β-AR-binding sites are found in cortical and limbic brain regions (Rainbow *et al.,* 1984). The putamen of the human brain was found to be rich in β_1-ARs, whereas the cerebellum contained predominantly β_2-ARs (Pazos *et al.,* 1985). The cellular localization of receptor-specific mRNA by *in situ* hybridization in the rat brain basically confirmed the results obtained with receptor-binding studies showing localizations of β_1- and β_2-AR gene expression in distinct neuroanatomical structures (Nicholas *et al.,* 1993b). However, there are species differences in β-AR distribution patterns in the brain. In the mouse, a stronger β-AR distribution pattern was detected in the superior colliculus compared to the rat (Lorton and Davis, 1987).

In the baboon brain, high numbers of β-ARs were detected in the cerebral cortex and the striatum with a preponderance of β_1-ARs, although in this autoradiographic receptor-binding study, nonspecific binding sites for the radioligand ^{125}I-iodocyanopindolol were not blocked so that part of the labeling may represent serotonergic receptors (Slesinger *et al.,* 1988). During aging, multiple alterations in β-AR densities occur in distinct brain regions, including increases and decreases in binding-site numbers (Weiland and Wise, 1986). These data, as well as the diffuse pattern of receptor distribution, led to the conclusion that β-ARs are also present on glia (Stone and John, 1991; Flügge *et al.,* 1997a; see Fig. 7).

The development of receptor subtype-specific antibodies made it possible to localize β-ARs in single cells and, in addition, using electron microscopy, to localize them on the subcellular level. In the nucleus of the solitary tract, at the border to the area postrema, β-AR-like immunoreactivity was found

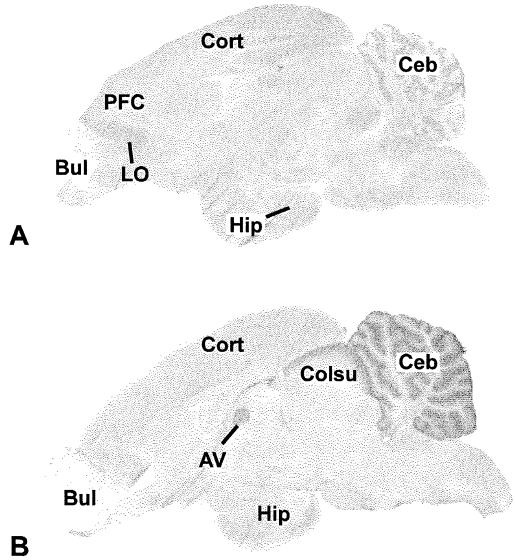

FIG. 7 The β-adrenergic receptor in the tree shrew brain visualized by binding of ^{125}I-iodo cyanopindolol in the presence of serotonin to block nonspecific binding. (A) β_1-AR binding (incubation of sections was performed in the presence of the antagonist ICI118.551 to block β_2-ARs). (B) β_2-AR binding (in the presence of the antagonist CGP20712A to block β_1-ARs; according to Flügge *et al.,* 1997a). Black areas demonstrate the presence of high numbers of receptors and white areas show no receptors. Autoradiograms show parasagittal sections approximately 3.0 (A) and 2.5 (B) mm lateral from the midline. Note the low binding and diffuse pattern, especially for β_1-ARs. AV, anteroventral thalamic nucleus; Bul, olfactory bulb; Ceb, cerebelum; Colsu, superior colliculus; Cort, cerebral cortex; Hip, hippocampal formation; LO, lateral orbital cortex; PFC, prefrontal cortex; Tha, thalamus.

along the intracellular surface of the plasma membrane of astrocytic processes, supporting the view that β-ARs are expressed by glia (Aoki and Pickel, 1992b). Neuronal pre- and postsynaptic membrane specializations of axodendritic junctions were also found to be immunoreactive for β-ARs (Aoki *et al.,* 1989). In the visual cortex of monocular monkeys, immunocytochemical detection of β-ARs showed a striped pattern, which indicated that receptor expression is increased in the cells forming the inactive columns of the visual cortex (Aoki *et al.,* 1994).

c. Central Nervous β-ARs in Physiology and Pathophysiology Peripheral effects of β-AR agonists and antagonists have attracted great scientific attention in the past because the stimulation of peripheral β-ARs leads to an increase in heart rate and contractility, vasodilatation, lipid mobilization, and decreased airway resistance. The use of β-AR antagonists in the treatment of human heart failure reduced mortality significantly in recent years (Heidenreich *et al.,* 1997). Brain β-ARs have not been studied that extensively, but it is clear that β-AR-stimulating agents exert multiple influences on neuronal and glial function. The earliest indication that the effects of noradrenaline on central neurons might be mediated by β-ARs arose from the finding that noradrenaline increased cAMP concentrations in brain slices (Kakiuchi and Rall, 1968). Radioligand-binding techniques were used to investigate the distribution and pharmacological characteristics of β-AR-binding sites in brain and spinal cord (Alexander *et al.,* 1975). In cortical pyramidal neurons, activation of β-ARs results in enhanced excitability and responsiveness to depolarizing inputs (McCormick *et al.,* 1991). In the hippocampus, a β-AR mechanism leads to long-term potentiation (Harlesy, 1991), and for the hippocampal region CA1 it was shown that the activation of presynaptic β-ARs enhanced synaptic transmission via activation of a cAMP-dependent protein kinase (Gereau and Conn, 1994). However, glial β-ARs may also play an indirect role in neurotransmission. In cultured spinal cord astrocytes, β-adrenergic receptor ligands modulate inward rectifier potassium channels via the activation of adenylate cyclase without involvement of PKA (Roy and Sontheimer, 1995). This mechanism desensitizes rapidly on chronic receptor stimulation as found in primary glial cultures, where prolonged agonist exposure led to a decline in cAMP accumulation (Atkinson and Minneman, 1992).

β-AR blockers are widely used in the treatment of psychiatric disorders such as anxiety, including the tendency for fear reactions in challenging situations when somatic symptoms are prominent (Bailly, 1996). Direct evidence that β-AR functioning is involved in emotional memory comes from a study showing that the β-AR blocker propranolol impairs memory of events that are accompanied by emotional arousal (Cahill *et al.,* 1994). The pathophysiology of depression may also be due in part to β-AR dys-

functioning. In several experimental models, chronic treatments with antidepressants caused the downregulation of β-ARs in the brain. Thus in slices from the rat cerebral cortex, prolonged administration of the tricyclic antidepressant desmethylimipramine (DMI) induced a 35–40% decrease in the accumulation of cAMP in response to isoproterenol stimulation, and this reduced receptor response was accompanied by a decrease in ^{125}I-iodohydroxypindolol binding (Wolfe *et al.,* 1978).

It was concluded from these experiments that the antidepressant acts presynaptically via β-ARs on noradrenergic terminals to increase the concentration of noradrenaline at the synapses, which then induces a compensatory decrease in the density of β-ARs on target neurons. As now known, DMI is primarily an inhibitor of noradrenaline reuptake, so that blockade of glial uptake sites, which raises extracellular noradrenaline, also has to be considered as a cause for the effects. Results showing that DMI induced β-AR desensitization mechanisms were later confirmed by similar studies demonstrating that administration of the drug to rats caused significant reductions in the binding of ^{125}I-iodocyanopindolol in many areas of the brain primarily representing reductions in β_1-AR numbers (Ordway *et al.,* 1988; Paetsch and Greenshaw, 1993). It could therefore be concluded that a β-adrenergic mechanism contributes to the beneficial effects of this antidepressant.

This assumption was confirmed by the finding that after lesioning noradrenergic fibers in the rat cerebral cortex, DMI induced regeneration of the noradrenergic axons and that this restorative effect of the antidepressant was blocked by the β-AR antagonist propranolol (Nakamura, 1994b). However, it is still unknown whether the desensitization of β-ARs contributes to the beneficial effects of the drug, especially since it has been reported that human depression itself is accompanied by a decrease in cortical β-ARs. In at least one study on suicide victims, the number of β-AR-binding sites in the frontal cortex turned out to be lower than in matched controls (Little *et al.,* 1993). Furthermore, chronic treatments of rats with other antidepressants such as citalopram and fluoxetine, which belong to the group of selective serotonin reuptake inhibitors (SSRIs), have been shown to increase ^{125}I-iodocyanopindolol binding to β_1-ARs in the somatosensory areas of the frontal cortex and the striatum (Palvimaki *et al.,* 1994).

B. Serotonin$_{1A}$-receptors

1. Molecular Biology and Pharmacology

The serotonin$_{1A}$-receptor (5HT$_{1A}$-receptor), a member of the highly conserved 5HT1 receptor family, is the best characterized 5HT receptor

(Sanders-Bush and Canton, 1995). Its human homolog consists of 421 amino acids (molecular mass approximately 46,000 Da) encoded by an intronless gene on chromosome 5. It contains three glycosylation sites on the extracellular part of the molecule and a disulfide bond between cysteine residues 109 and 186 (DeVivo and Maayani, 1986; Watson and Arkinstall, 1994). Stimulation of the receptor reduces adenylate cyclase activity, activates potassium channels, and inhibits opening of calcium channels through a pertussis toxin-sensitive G-protein of the G_i/G_o class (Albert and Morris, 1994). When expressed in certain cell lines, stimulation of the receptor does not reduce basal cAMP levels but instead stimulates phospolipase C activity (Hamon *et al.,* 1990). In the brain, there are regional differences in $5HT_{1A}$-receptor coupling to second messenger systems. Thus, in the hippocampus, receptor stimulation inhibited forskolin-stimulated adenylate cyclase activity, whereas in the dorsal raphe nucleus, phosphoinositide hydrolysis was inhibited (Johnson *et al.,* 1997).

Using the highly specific and stable agonist 8-OH-DPAT (8-hydroxy-2-[di-n-propylamino]tetralin), the $5HT_{1A}$-receptor has been studied extensively (Vergé *et al.,* 1986). The antagonist WAY-100635, which became available in the 1990s also turned out to be selective for the $5HT_{1A}$-receptor, whereas other ligands that had formerly been used for characterization of $5HT_{1A}$-receptor binding, such as pindolol, are nonselective and also label other monoamine receptors (Forster *et al.,* 1995; Khawaja, 1995; Hamon *et al.,* 1986). The azapirones buspirone and ipsapirone, which have beneficial effects in the clinical treatment of anxiety and mood disorders, act as partial agonists (Coplan *et al.,* 1995; Blier and Montigny, 1998).

2. Physiological Role

Due to its ablity to open potassium channels and close L-type calcium channels, the $5HT_{1A}$-receptor can hyperpolarize neuronal membranes and thus modulate neuronal activity (Sanders-Bush and Canton, 1995). In this way, firing of serotonergic neurons in the dorsal raphe nucleus, which carry large numbers of these ''autoreceptors,'' is inhibited by agonists such as 5HT and 8-OH-DPAT, but stimulated by the antagonist WAY-100135 (Gozlan *et al.,* 1983; Hamon *et al.,* 1990; Mundey *et al.,* 1994). Serotonin release and turnover are also inhibited when the somatodendritic $5HT_{1A}$-autoreceptors in the raphe nuclie are stimulated. In the respiratory network of the brain stem, certain respiratory neurons are inhibited by $5HT_{1A}$-receptor activation, which, in the cat, suppresses apneusis (Lalley *et al.,* 1994, 1997). Differential effects of 5HT on neuronal activity occur in the hippocampus, where interneurons are regulated via $5HT_{1A}$-receptors (Schmitz *et al.,* 1995). Stimulation of postsynaptic $5HT_{1A}$-receptors in the hippocampus elicits hyperpolarization and a decrease in the membrane

resistance of CA1 pyramidal neurons (Joëls and DeKloet, 1994). For the hippocampal region CA3, it has been shown that $5HT_{1A}$-receptors are linked to an inward-rectifying potassium channel (Okuhara and Beck, 1994). Furthermore, the spike responses of dentate granular cells to perforant path stimulation are modulated by $5HT_{1A}$-receptor-regulated interneurons (Levkovitz and Segal, 1997).

Neurons that produce serotonin innervate the autonomic centers in the brain. Because neurons of the descending serotonergic system project to autonomic areas of the lower brain stem and spinal cord and because the ascending serotonergic system includes forebrain cardiovascular centers, one could assume that 5HT-receptors are involved in cardiovascular regulation. Indeed, it was found that activation of $5HT_{1A}$-receptors in the hindbrain has sympathoinhibitory effects, probably by blocking sympathetic nerve activity and enhancing activity of the cardiac vagal nerve. In contrast, forebrain areas appear to be sites for sympathoexcitatory responses to $5HT_{1A}$ agonists (McCall and Clement, 1994).

Sleep is known to be modulated by the serotonergic system and there are indications that $5HT_{1A}$-receptors are intimately involved in this process. In the cat, administration of 8-OH-DPAT to dorsal raphe neurons by microdialysis increased rapid eye movement (REM) sleep while decreasing serotonin release (Portas *et al.,* 1996). Data from animal experiments are consistent with the hypothesis that reduced 5HT neurotransmission following $5HT_{1A}$-autoreceptor stimulation in the dorsal raphe nucleus disinhibits cholinergic mesopontine neurons, which leads to the increase in REM sleep (Bjorvatn *et al.,* 1997). Possibly related to the $5HT_{1A}$-receptor-mediated alterations in sleep patterns are changes in circadian rhythms of the receptors, which have been observed in Syrian hamsters and other species (Hulihan-Giblin *et al.,* 1993; Lu and Nagayama, 1997; Mintz *et al.,* 1997). Furthermore, there are other physiological processes that are supposed to be regulated via $5HT_{1A}$-receptors, such as thermoregulation and nociception (Goodwin *et al.,* 1985; Hamon *et al.,* 1990).

The neuronotrophic actions of serotonin may also be mediated by $5HT_{1A}$-receptors. The partial agonist ipsapirone at least displayed a trophic influence on primary cultures of septal cholinergic neurons by altering the morphology of their neuritic tree (Riad *et al.,* 1994). In cultures of hippocampal cells, ipsapirone raised the number of synaptophysin immunoreactive varicosities, which indicates an increase in synapse formation (Nishi *et al.,* 1996).

3. $5HT_{1A}$-Receptor Regulation

Like other G-protein-coupled receptors, $5HT_{1A}$-receptors are desensitized on sustained exposure to agonists. Thus, chronic treatment with 8-OH-

DPAT has been reported to lead to receptor desensitization (Kreiss and Lucki, 1992). Naturally occurring variants of the human $5HT_{1A}$-receptor with mutations at distinct sites of the receptor molecule may display reduced sensitivity to such agonist-mediated desensitization (Rotondo *et al.,* 1997). However, agonist-mediated signals may not play a prominent role in the regulation of $5HT_{1A}$-receptor expression in the brain because a deficit in serotonin induced by chemical disruption of the dorsal raphe nucleus did not lead to changes in expression of the $5HT_{1A}$-receptor gene in the hippocampal formation and other brain areas (Henseler *et al.,* 1991; Miquel *et al.,* 1992). Instead, expression of these receptors is clearly regulated by steroid hormones. Due to their lipophilicity, these hormones penetrate the blood–brain barrier and thus reach cells in the brain where they can exert their so-called genomic effects, meaning that they regulate the transcription of numerous genes via intranuclear steroid receptors that bind to the promotor regions of those genes (Beato *et al.,* 1996). Glucocorticoids that are secreted from the adrenal cortex (corticosteroids) exert their influence primarily via two receptors: the mineralocorticoid receptor (MR), which displays a high affinity for corticosterone (the dominating circulating glucocorticoid in the rat), and the glucocorticoid receptor (GR), which has only low affinity for corticosterone. Glucocorticoids induce downregulation of the $5HT_{1A}$-receptor (Mendelson and McEwen, 1992; Chalmers *et al.,* 1993; Chaouloff, 1993). After adrenalectomy in rats, when there is a lack of adrenal hormones in the circulation, mRNA for $5HT_{1A}$-receptors in the hippocampal formation is upregulated by more than 30% (Zhong and Ciaranello, 1995). This change in amounts of mRNA is also reflected in numbers of ligand-binding sites (Tejani-Butt and Labow, 1994). However, there are regional differences in $5HT_{1A}$-receptor regulation. Whereas chronic stress, which is accompanied by high levels of corticosteroids, downregulated these receptors in several areas of the frontal brain, receptor numbers did not change in the dorsal raphe nucleus (Flügge, 1995).

Effects of corticosteroids on $5HT_{1A}$-receptors have consequences for the electrical activity of the neurons. Using electrophysiological methods, it was found that corticosterone exerts its influence on $5HT_{1A}$-receptors primarily via the MR (Joëls and DeKloet, 1994). Activation of MR suppresses $5HT_{1A}$-receptor-mediated neuronal hyperpolarization in region CA1 of the hippocampus, whereas activation of GR enhances the effect of 5HT and hyperpolarizes cells (Joëls *et al.,* 1991). The low-affinity GR is only activated when there are high concentrations of the glucocorticoid such as during stress. Indirect evidence for the hypothesis that the MR is responsible for glucocorticoid-induced regulation of $5HT_{1A}$-receptors under normal physiological conditions was obtained from studies on GR-deficient transgenic mice showing no deficit in the glucocorticoid-induced regulation of $5HT_{1A}$-receptors (Meijer *et al.,* 1997). In addition to corticosteroids, the sex

steroid estradiol has also been reported to downregulate $5HT_{1A}$-receptor message in the dorsal raphe nucleus of rhesus macaques, and an acute estradiol treatment decreased the amount of mRNA in the limbic system of the female rat (Pecins-Thompson *et al.,* 1996; Österlund and Hurd, 1998). However, these experimentally induced ''negative'' effects of the sex hormone do not necessarily reflect the normal physiological situation, because our own data from male animals showed that the downregulating effect of stress on $5HT_{1A}$-receptors in the hippocampal formation can be counteracted by a treatment with testosterone, indicating an upregulatory effect of this sex steroid (Flügge *et al.,* 1998; see later). Furthermore, we found that the number of $5HT_{1A}$-receptors in the ventromedial hypothalamic nucleus (a nucleus involved in the regulation of sexual behavior) in intact cycling female rats is increased slightly during the time when estradiol levels are high (Flügge *et al.,* 1999). However, other factors may also have an impact on regulation of these receptors. Curiously enough, an extract from the *Ginkgo biloba* plant has been reported to regulate $5HT_{1A}$-receptor numbers in the brain. In aged rats, the numbers were decreased but the plant extract brought them back to baseline (Huguet *et al.,* 1994). Whether this effect might be secondary to endocrinological changes in the animals or to an unknown compound in the extract acting directly on the $5HT_{1A}$-receptor system is not known.

4. Neuroanatomical Distribution of $5HT_{1A}$-Receptors

The pattern of $5HT_{1A}$-receptors in the brain has been largely mapped by *in vitro* autoradiography using [^{3}H]8-OH-DPAT as the radioligand. In several species, a strong binding was detected in the raphe nuclei, representing the somatodendritic autoreceptors, and in the hippocampal formation, representing postsynaptic receptors. Moderate numbers of binding sites were found in cortical and other regions (Palacios *et al.,* 1990; Flügge 1995; Stockmeier *et al.,* 1996; see Fig. 8). However, different species show different distribution patterns of receptors in the brain. In the rat, hippocampal region CA1 and dentate gyrus display almost equal amounts of [^{3}H]8-OH-DPAT-binding sites, whereas in the tree shrew brain, labeling in the dentate gyrus is much weaker than labeling in region CA1 (Flügge *et al.,* 1998). A species difference in occurrence of $5HT_{1A}$-receptors has been reported for the septum, where rats showed high receptor numbers but the human brain revealed low receptor binding (Duncan *et al.,* 1998). Detailed anatomical mappings of [^{3}H]8-OH-DPAT-binding sites were performed in the tree shrew brain. In the amygdala, a high number of $5HT_{1A}$-receptor-binding sites was detected in the magnocellular subdivision of the basal nucleus (Flügge *et al.,* 1994). In the anterior olfactory nucleus, a strong [^{3}H]8-OH-DPAT binding was also observed, and this labeling extends into the tenia

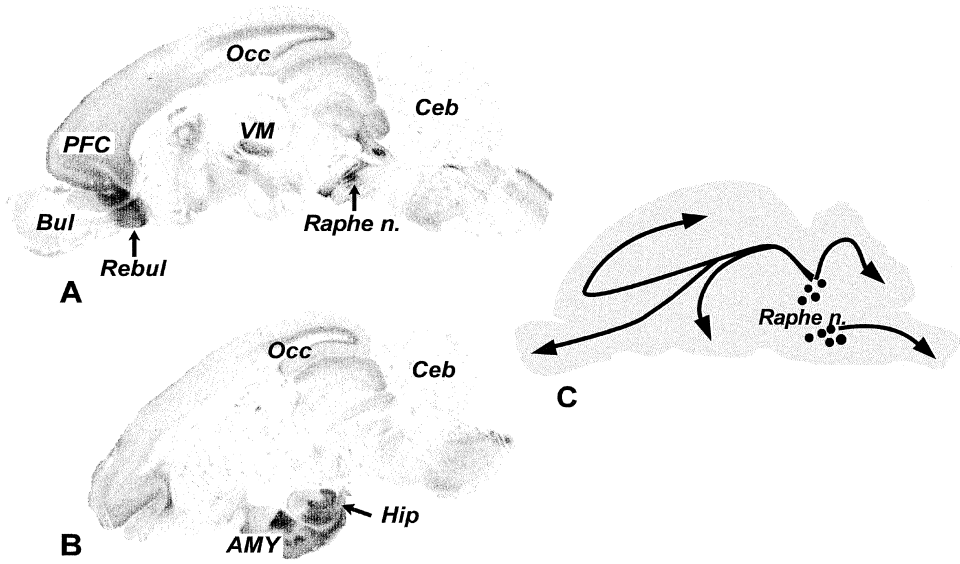

FIG. 8 Serotonin$_{1A}$-receptors in the tree shrew brain visualized by receptor autoradiography with the radioligand [^{3}H]8-OH-DPAT (A, B). Black areas demonstrate the presence of high numbers of receptors and white areas show no receptors. Note the patches of high numbers of receptors in the raphe nuclei (Raphe n.), in the ventromedial thalamic nucleus (VM), the amygdala (AMY), and the hippocampal formation (Hip). Sagittal sections are cut through the midline of the brain (A) and 4 mm lateral from the midline (B). (C) Serotonergic projections from raphe nuclei to different parts of the brain. Bul, olfactory bulb; Ceb, cerebellum; Occ, occipital cortex; PFC, prefrontal cortex; Rebul, retrobulbar region.

tecta and the orbital cortex (Flügge, 1995). Furthermore, in the prefrontal cortex, binding of [^{3}H]8-OH-DPAT generates distinct bands that do not exactly match the cortical layers visualized by Nissl staining (Flügge *et al.,* 1997c). Prefronto-cortical layer I is strongly labeled and this labeling reaches into layer II. The moderate labeling of layer IV extends into layer III. In the occipital cortex, an area that overlaps with the visual cortex, a prominent [^{3}H]8-OH-DPAT labeling is observed in layers V and VI. As found in the tree shrew by autoradiographic receptor-binding studies, 5HT$_{1A}$-receptors are present in the thalamus, close to the medullary lamina and in the ventromedial thalamic nucleus (Flügge, 1995; Flügge *et al.,* 1998). In the hypothalamus of the rat, a high number of receptors was detected in the ventromedial nucleus, which is involved in the regulation of female sexual behavior (Flügge *et al.,* 1999). This distribution of 5HT$_{1A}$-receptors has been confirmed by immunocytochemical experiments using a specific antibody raised against the third intracellular loop of the receptor molecule (Kia *et al.,* 1996b). Furthermore, this receptor distribution was confirmed by *in situ*

hybridization experiments and, in addition, showed no indications for a glial localization of $5HT_{1A}$-receptor expression (Burnet *et al.,* 1995; Mengod *et al.,* 1997). However, it has been discussed whether the receptors might be expressed by glial cells (Azmitia *et al.,* 1996). On the ultrastructural level, $5HT_{1A}$-receptor immunoreactivity was almost exclusively found in the somatodendritic compartment of neurons and very rarely in processes belonging to glial cells (Kia *et al.,* 1996a). A possible explanation for discrepancies in results might be the characteristics of the antipeptide antibodies used in these studies, which appeared to have different affinities for different molecular conformations of the receptor (Anthony and Azmitia, 1997).

5. $5HT_{1A}$-Receptors and Behavior

Serotonin receptors have been implicated in the regulation of mood and emotional behavior, and several experimental setups have been created to demonstrate this by evaluating animal behavior (Lucki, 1992). The anxiolytic-like properties of $5HT_{1A}$-receptor agonists can be recognized as changes in avoidance behavior of animals. Thus, in rats, a subcutaneous injection of 8-OH-DPAT produced behavioral effects in a defense test system that are consistent with reduced anxiety and fear (Blanchard *et al.,* 1992). The agonist ipsapirone dose dependently reduced defensive behavior and increased the amount of time spent on feeding behavior (Korte *et al.,* 1992). Data indicate that the site of anxiolytic actions of the drug is the dorsal raphe nucleus and that inhibition of 5HT release evoked by stimulation of the somatodendritic autoreceptor leads to the anxiolysis. Thus, injection of 8-OH-DPAT directly into the dorsal raphe nucleus of rats prevented the fear response to inescapable foot shock (Maier *et al.,* 1995). In a social interaction test and in the ''elevated plus maze test'' for anxiety, 8-OH-DPAT injections into the raphe nuclei also produced anxiolysis, whereas injections into the hippocampus, and thus stimulation of postsynaptic receptors, resulted in anxiogenic reactions (File *et al.,* 1996). Furthermore, anxiogenic-like reactions in a social interaction test were observed in rats with implants in the amygdala containing 8-OH-DPAT (Gonzalez *et al.,* 1996). Finally, an involvement of $5HT_{1A}$-receptors in anxiety reactions has been suggested by the finding that rat strains that differ in their hypothermic reaction to $5HT_{1A}$ agonists also showed strain-specific anxiogenic reactions when 8-OH-DPAT was injected into the hippocampus (Gonzalez *et al.,* 1998). In mice, the behavioral effects of $5HT_{1A}$-receptor agents are more difficult to interpret. Activation of $5HT_{1A}$ receptors by agonists injected intraperitoneally induced behavioral changes associated with the 5HT syndrome (Blanchard *et al.,* 1997). These include alterations in the locomotion pattern initially after the injection, followed by hyperactivity after about 1 h. Consistent with the view that hyperactivity of the 5HT

system results in increased anxiety are data from genetically engineered mice without a $5HT_{1A}$-receptor, which probably have an impaired regulation of neuronal 5HT release. These mice showed an increased tendency to avoid a novel, fear-inducing environment (Parks *et al.,* 1998).

A brain region where $5HT_{1A}$-receptor agonists might modulate aversive behavior is the periaqueductal gray (Nogueira and Graeff, 1995). In rats, 8-OH-DPAT counteracted the explosive motor behavior induced by injections of an excitatory amino acid into this brain region. In contrast, the antagonist WAY-100635 potentiated the intensity of the aversive reaction (Beckett and Marsden, 1997). In the cat, defensive rage behavior (hissing) elicited by electrical stimulation of the medial hypothalamus was counteracted by 8-OH-DPAT injections into the periaqueductal gray (Shaikh *et al.,* 1997). However, there are some discrepancies in results on $5HT_{1A}$-receptor-regulated behavior. Some authors have suggested that pharmacological variables, such as the route of administration or the doses used, may account for this. Of course, differences in test systems may also influence behavioral data. From the large number of data one has to conclude that behavioral effects of $5HT_{1A}$-receptor ligands can switch easily from ''anxiolytic'' to inactive or even ''anxiogenic'' when alterations in the experimental procedure are performed (Griebel, 1995). Thus in mice, the $5HT_{1A}$-receptor antagonist p-MPPI produced a dose-related anxiolytic profile in avoidance tests when low doses of the drug were injected. At high doses, the effect was lost and the animals increased grooming and immobility (Cao and Rodgers, 1997). Furthermore, gender-specific behaviors are also modulated by $5HT_{1A}$-receptor agonists. Activation of $5HT_{1A}$-receptors in the ventromedial hypothalamic nucleus of female rats inhibits lordosis behavior, and male sexual behavior in animals primed with testosterone is also facilitated by receptor agonists (Jackson and Uphouse, 1996; Mendelson and Gorzalka, 1986). In summary, although there is some variability in reports about the behavioral effects of 8-OH-DPAT, anxiolytic-like properties of buspirone and 8-OH-DPAT have been demonstrated in the majority of publications (Griebel, 1995).

6. $5HT_{1A}$-Receptors and Disease

Because depression is supposed to be induced by alterations in the serotonergic system, $5HT_{1A}$-receptor functioning may also play a role in this disorder. There are three lines of evidence indicating that $5HT_{1A}$-receptors are indeed involved in central nervous mechanisms of depression: (1) The $5HT_{1A}$-agonists buspirone and gepirone had antidepressant-like effects in clinical trials (Blier and Montigny, 1998). These drugs are supposed to decrease 5HT release from the raphe nuclei, which, after prolonged treatment, might be followed by $5HT_{1A}$-autoreceptor desensitization coinciding

with gradual recovery from clinical symptoms. (2) Data from animal studies showed that chronic social stress, which leads to depressive-like behavior, downregulates $5HT_{1A}$-receptors in the hippocampal formation and distinct cortical structures (Flügge, 1995; McKittrick *et al.,* 1995). (3) Postmortem studies on the human brain showed that the $5HT_{1A}$-receptor number in the hippocampal formation of suicide victims is lower than in matched controls (Lopez *et al.,* 1998). However, there are conflicting data on this issue. In an older study, nonviolent suicides displayed significantly higher B_{max} values for [^{3}H]8-OH-DPAT binding in the frontal cortex compared to controls and violent suicides, and another report showed no difference in $5HT_{1A}$-receptor binding in various brain regions of suicides and controls (Matsubara *et al.,* 1991; Lowther *et al.,* 1997). For the human frontal cortex, it was found that it is predominantly the ventrolateral part where $5HT_{1A}$-receptor-related dysfunctions occur (Arango *et al.,* 1995). In line with the hypothesis that low $5HT_{1A}$-receptor numbers in the hippocampal formation are related to depression are animal studies showing that antidepressants such as imipramine renormalize the number of these receptors in stressed rats (Lopez *et al.,* 1998). Chronic injections of the serotonin uptake inhibitor fluoxetine reduced the response of the hypothalamic–pituitary–adrenal (HPA) axis to a challenge with 8-OH-DPAT, indicating low $5HT_{1A}$-receptor sensitivity (Li *et al.,* 1994).

Furthermore, fluoxetine reduced the potency of a $5HT_{1A}$-receptor agonist to inhibit the firing of serotonergic neurons, which also indicates low $5HT_{1A}$-receptor sensitivity (Lepoul *et al.,* 1997). Interestingly, this mechanism appears to be independent from glucocorticoids, which coincides with our own finding that $5HT_{1A}$-receptors in the dorsal raphe nuclei are regulated in a different way than the hippocampal receptors that are downregulated by glucocorticoids. However, another selective 5HT reuptake inhibitor, citalopram, did not desensitize the autoreceptors in the dorsal raphe nucleus (Hjorth and Auerbach, 1994). Furthermore, data show that several steps in the regulatory pathway still need to be elucidated. In slices from the dorsal raphe nucleus of rats treated with the serotonin transport inhibitor paroxetine, the $5HT_{1A}$-agonist-induced firing response was decreased corresponding to desensitized autoreceptors, whereas, surprisingly, the capability to control 5HT release was enhanced (Davidson and Stamford, 1998). On the basis of results from a 3-week treatment with the $5HT_{1A}$-receptor agonist ipsapirone it was concluded that dosage and length of the treatment are important experimental parameters for $5HT_{1A}$-receptor determinations (Fanelli and McMonagle-Strucko, 1992). This statement is confirmed by our own observation that [^{3}H]8-OH-DPAT-binding sites are reduced by 10% in the dentate gyrus 3 h after a single intravenous injection of a physiological sodium chloride solution (unpublished observations).

Central nervous disorders other than depression may also be related to changes in central nervous $5HT_{1A}$-receptors. A variant of the $5HT_{1A}$-receptor gene was found in Tourette's patients, and in schizophrenics, [^{3}H]8-OH-DPAT-binding sites were increased in the ventral prefrontal cortex (Lam *et al.,* 1996; Simpson *et al.,* 1996; Sumiyoshi *et al.,* 1996). Whereas in the normal adult human brain, the cerebellum showed low or even no [^{3}H]8-OH-DPAT binding, schizophrenics displayed some $5HT_{1A}$-receptor binding in this brain region, indicating that the postnatal regression of cerebellar receptors is slowed or even arrested in the patients (Slater *et al.,* 1998). The mechanisms that might underlie $5HT_{1A}$-receptor malfunctioning in the human brain are unclear at present. Up until now there have been no indications that the dysregulations of the receptor might be due to alterations in the receptor gene (Xie *et al.,* 1995).

IV. Stress and the Tree Shrew Paradigm

Stress is a common experience of daily life and all organisms have evolved mechanisms and strategies to deal with alterations in their internal and external environment. The potency to cope with and adapt to stressors is a fundamental requirement for survival. In vertebrates, diverse stressors activate a wide spectrum of neuronal and hormonal systems, resulting in physiological and behavioral responses. These stress responses are variable and there are individual differences both physiologically and behaviorally in how an organism perceives a perturbation and how it adapts to a new situation (Henry and Stephens, 1977; McEwen, 1994; Sapolsky, 1994). The ability of a brain to perform functionally relevant adaptations following various challenges is called plasticity. Neuronal plasticity is absolutely necessary for adequate functioning of an individual in a continuously changing environment. The underlying dynamic processes are based on the capability of transmitter systems, brain nuclei, single neurons, synapses, and receptors to adapt and modify specific structures and functions (Fillenz, 1993). Central nervous adaptive plasticity reveals itself in many forms, ranging from changes in neurotransmitter release, to alterations in expression of neurotransmitter receptors, to changes in neuronal morphology.

In the brain, the activation of monoamine systems is a major component of the stress response. Stress-induced changes in the central nervous monoamine system are of special interest because the hyperactivity of catecholaminergic and serotonergic neurons that occurs during stress may lead to psychopathologies such as anxiety disorders and depression (Johnston, 1991; Stanford, 1993; Holsboer, 1995). Stress-induced alterations in the monoamine system are regarded as a basis for stress-related behavior

(Weiss *et al.,* 1981; Meerlo *et al.,* 1997). As demonstrated already in the 1960s, release and turnover of monoamines are increased during stressful experiences (Thierry *et al.,* 1968; Bliss *et al.,* 1968). Within the noradrenergic system, the LC plays an important role in mediating the stress response, and its particular reactivity to diverse stressful stimuli is well documented (Bremner *et al.,* 1996a). A high stress-induced neuronal activity in the LC has been demonstrated by electrophysiology, as well as biochemistry, revealing increased expression of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis (Segal, 1979; Smith *et al.,* 1991). Increases in the release of noradrenaline in the regions of noradrenergic terminal fields has also been documented (Nisenbaum *et al.,* 1991; Stanford, 1993). In addition to LC, medullary catecholamine neurons also play an important role in stress responses because they are involved in the regulation of basal autonomic functions such as cardiovascular control (Saavedra, 1988; Kubo *et al.,* 1990). In the serotonergic system, stress leads to elevated concentrations of serotonin and its metabolites in several brain regions, indicating increased turnover rates (Thierry *et al.,* 1968; Raab, 1971; Hellhammer *et al.,* 1984). As described earlier, the firing frequency of serotonergic neurons in the dorsal raphe nucleus depends on the animal's state of motor arousal, with no apparent decline in firing frequency on repetition of the stimulus (Jacobs and Fornal, 1995). Stress such as restraint or exposure to a predator increases single unit activity in the dorsal raphe nucleus, and although this increase is not larger than that observed during behavioral arousal, it is clear that stressful stimuli elevate 5HT release in the fields of serotonergic terminals.

Research from a variety of disciplines has emphasized the interrelationships between acute and chronic stress and the pathogenesis of mental illness such as anxiety disorders and depression (Holsboer 1995; Stout and Nemoroff, 1994). In animal models, stressors mostly involve noxious stimuli or perturbations of the physical environment such as electric foot shock, tail pinch, forced swimming, physical restraint, water and food deprivation, and cold exposure (Sutanto and deKloet, 1994). In contrast, common stressors in humans often include a psychological component. To bridge the gap between experimental models and the situation in humans, there is a need to use more psychological types of stressors in animal studies. In recent years, new powerful animal models using chronic psychosocial perturbations as stressors were established (Blanchard *et al.,* 1990). The psychosocial stress paradigm in male tree shrews (*Tupaia belangeri*) is an experimental model whose value for research on stress-induced changes in the CNS has been elaborated (Fuchs and Flügge, 1995; Fuchs *et al.,* 1996; Fuchs and Flügge, 1998). From the phylogenetic point of view, the day active tree shrew is regarded as an intermediate between insectivores and primates, combining features of both orders (Martin, 1990). The pronounced territori-

ality of males from this species can be used to establish naturally occurring challenging situations under experimental control in the laboratory. Because of their territoriality, adult male tree shrews normally have to be kept singly in their cages. If two males have to share one cage, which represents a territory, there is a short fight between the animals after which a clear social rank order is established with a dominant and a subordinate male. Under experimental conditions, physical contact between the individuals is allowed for only 1 to 2 h in the morning, while the rest of the day animals are separated by a wire mesh. As long as visual and olfactory communication is possible, the subordinate animal ''interprets'' the situation as the existence of a high level of dominant threat along with an unpredictable possibility of physical attacks.

This situation can be maintained over several days or even weeks, thus exposing the subordinate to chronic stress due to repetitive challenging confrontations with the dominant. As a consequence, subordinate animals show profound changes in physiological and behavioral mechanisms that are based exclusively on the cognitive interpretation of the continuous visual presence of the dominant conspecific (Raab, 1971; von Holst *et al.,* 1983). Behavioral analysis showed that subordinates reduce their locomotor activity and cease self-grooming and scent-marking behavior corresponding to a symptomatology, which is comparable to the symptoms observed in depressed psychiatric patients (Fuchs *et al.,* 1996; Brown, 1993). Their circadian rhythm is profoundly disturbed, leading to changes in the sleeping pattern with increased numbers of early waking episodes in the second half of the night and an elevation in heart rate (Fuchs and Schumacher, 1990; Stöhr, 1986). The sympathetic nervous system is activated constantly during periods of chronic psychosocial stress reflected by an increased urinary excretion of noradrenaline (Fuchs *et al.,* 1993). In relation to this, an elevated metabolic rate was observed, which is accompanied by diminished food and water intake, resulting in a significant decrease in body weight (Fuchs and Kleinknecht, 1983; Jöhren *et al.,* 1991). Analysis of endocrine parameters in subordinates revealed a constant hyperactivity of the HPA (hypothalamic-pituitary-adrenal) axis as demonstrated by high concentrations of urinary glucocorticoid hormones (Fuchs *et al.,* 1993). This hypercortisolism is paralleled by increased adrenal weights and reduced activities of gonads (Fuchs *et al.,* 1993; Fischer *et al.,* 1985). An advantage of the tree shrew model is that physiological responses to stress can be evaluated in individual animals over a long time period by determining hormones in morning urine over several weeks. The central nervous activation of monoamine systems in subordinate tree shrews has been demonstrated for the noradrenergic and the serotonergic system by the analysis of transmitter turnover and alterations in receptors (Raab, 1971; Raab and Storz, 1976; Flügge *et al.,* 1992).

A. α_2-AR Changes under Chronic Stress in Different Brain Areas

Older studies show that stress influences the numbers of α_2-adrenoceptors. Immobilization of rats lowered the number of midbrain and brain stem binding sites for the agonist [^{3}H]clonidine (U'Prichard and Kvetnansky, 1980), and acute cold stress decreased binding site numbers for the antagonist [^{3}H]rauwolscine (Nukina *et al.,* 1987). To see whether chronic stress also has an impact on α_2-ARs, we studied [^{3}H]rauwolscine binding in the tree shrew brain after 10 days of psychosocial stress (PSS). High number of [^{3}H]rauwolscine-binding sites were detected in solitary tract nucleus, dorsal motor nucleus of vagus, LC (250–300 fmol/mg), and in hypothalamic and amygdaloid regions (180 bis 220 fmol/mg), and moderate numbers in other regions, including the cortex (100–150 fmol/mg). It turned out that PSS affected these receptors in different regions: In 5 of the 14 brain areas investigated, subordinate tree shrews had significantly lower numbers of [^{3}H]rauwolscine binding sites (up to 20%) than their dominant counterparts (Flügge *et al.,* 1992). These brain structures were the solitary tract nucleus, the dorsal motor nucleus of the vagus, the periaqueductal gray, the perifornical region of the hypothalamus, and the medial nucleus of the amygdala, areas that are all intimately involved in the regulation of autonomic functions and emotional behavior.

It is likely that the decrease in α_2-AR numbers in subordinates affects the functions of these brain areas. (1) The nucleus of the solitary tract receives afferents from visceral nerves, including the vagal nerve, and serves as a relay station for the peripheral baroreceptor inputs (Kalia, 1981). α_2-ARs in this nucleus seem to play a part in mechanisms that regulate blood pressure, as it has been observed that hypertensive rats have different α_2-AR characteristics than normotensive rats and that catecholamines influence the baroreceptor reflex via α_2-ARs in this nucleus (Nomura *et al.,* 1985; Kubo *et al.,* 1990). Because heart rate is also modulated by α_2-ARs in the solitary tract nucleus, the low number of [^{3}H]rauwolscine-binding sites in subordinates may be related to the fact that subordinates show dramatic elevations in heart rate during periods of daily social interactions between two male conspecifics (Tung *et al.,* 1988; Stöhr, 1986). (2) The dorsal motor nucleus of the vagus contains preganglionic cells that innervate the parasympathetic ganglia of the thoracic and abdominal organs. Alterations in α_2-AR expression may therefore have an impact on the control of functions of these visceral organs. (3) The periaqueductal gray has extensive afferent and efferent projections and thus modulates many brain functions, including the regulation of defensive behavior, analgesia, and autonomic reactions (Andrezik and Beitz, 1985; Lovick, 1993; Bandler and Shipley, 1994). (4) Furthermore, the perifornical region of the hypothalamus is known to integrate the control of autonomic responses associated with

emotional reactions (Smith and Devito, 1984). When this brain region is stimulated in a dominant male baboon, the animal immediately attacks a conspecific. Adrenergic receptors in the perifornical region are probably involved in these integrative processes, which may be impaired in subordinate tree shrews with reduced α_2-AR numbers. A further relation to stress regulation is indicated by data showing that hypothalamic α_2-ARs regulate the release of the corticotropin-releasing factor, a major component of the endocrine stress response (Haas *et al.,* 1990). α_2-AR changes in the hypothalamus may therefore contribute to the peripheral reactions observed in subordinates, especially to the increase in activity of the hypothalamic–pituitary–adrenal axis. (5) In view of the well-known role of the amygdala in emotional events, it can be assumed that the stress-mediated changes in α_2-ARs in the medial amygdaloid nucleus have an impact on emotional reactions (LeDoux, 1995).

Because it is known that stress raises the activity of noradrenergic and adrenergic neurons, thus increasing the release and turnover of nor/adrenaline, it is probable that the decrease in the number of α_2-adrenergic-binding sites represents downregulation of α_2-ARs related to the high noradrenergic activity. Downregulation of α_2-ARs is a common phenomenon that has been studied *in vitro* with cell lines expressing human α_2-AR subtypes, showing that the loss in membrane receptors in the continuous presence of agonists leads to desensitization of the cellular signaling system (Eason and Liggett, 1992). The fact that subordinates had lower receptor numbers than dominants indicates that subordinates do not have the regulatory mechanisms to cope with challenging situations. In the social context, this state of the noradrenergic system with low expression of α_2-ARs might constitute the disposition to react in a defensive way to social encounters (Haller *et al.,* 1995).

Both numbers and affinities of [^{3}H]rauwolscine-binding sites in subordinates differed from those in the dominants (Flügge *et al.,* 1992). In three nuclei, the solitary tract nucleus, the periaqueductal gray, and the medial nucleus of the amygdala, dominants showed significantly lower affinities for the α_2-AR antagonist than subordinates, indicating the presence of a low-affinity binding site in dominants that is not present in subordinates. Because it is now known that the antagonist rauwolscine has a higher affinity for the α_2-AR subtype C compared to subtype A, the difference in affinity for [^{3}H] rauwolscine may be due to a preponderance of subtype A receptors in dominants, whereas in subordinates, this subtype is downregulated (MacDonald *et al.,* 1997; see later).

1. Time Course of α_2-AR Changes

Because periods of PSS can last for several days or even weeks in the tree shrew model, we analyzed whether changes in α_2-ARs show a stress–time-

dependent pattern (Flügge, 1996; see Fig. 9). Male tree shrews were submitted to PSS for 2, 10, 21, and 28 days, respectively, and on the following day, approximately 24 h after the last confrontation with the dominant, brains were dissected and α_2-AR-binding characteristics were determined by *in vitro* receptor autoradiography using the antagonist [^{3}H]RX821002. This technique allows quantification of receptor numbers and affinity in distinct nuclei of the brain (Fig. 3). Molecular biology experiments with cell lines expressing selective α_2-AR subtypes had shown that [^{3}H]RX821002 binds with almost equal affinity to subtype A (K_d = 0.48 n*M*) and subtype C (K_d = 0.63 n*M*) and with lower affinity to subtype B (K_d = 1.83 n*M*; Halme *et al.,* 1995). The overall autoradiographic pattern of [^{3}H]RX821002 labeling in the tree shrew brain resembles that of [^{3}H]rauwolscine with high numbers of ligand-binding sites in brain stem nuclei and many regions of the frontal brain (Flügge, 1996). Chronic stress

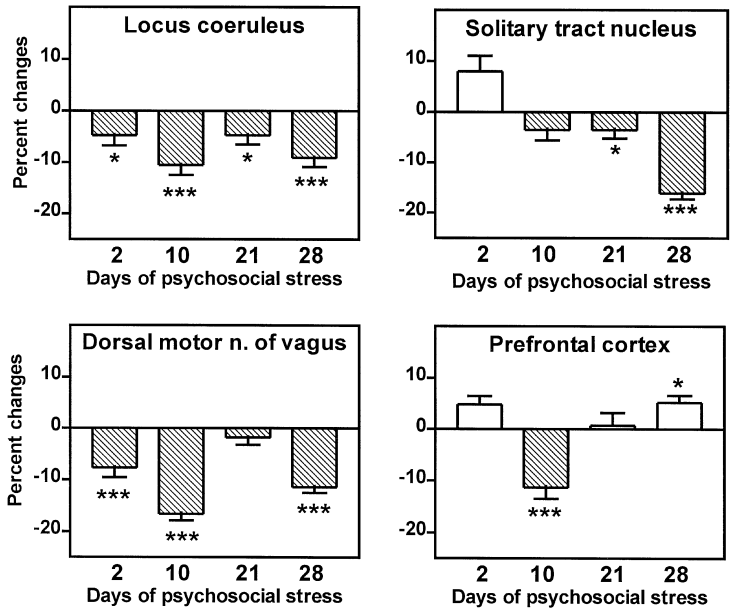

FIG. 9 Changes in α_2-adrenoceptorr numbers in the brains of male tree shrews chronically exposed to psychosocial stress for 4 weeks. Receptor numbers were determined by *in vitro* receptor autoradiography with the radioligand [^{3}H]RX821002 and expressed as percentage of receptor numbers in control animals. Note that α_2-adrenoceptos in the locus coeruleus, which represent the autoreceptors on the noradrenergic neurons, are already downregulated after 2 days of psychosocial stress and then stay low during the whole stress period. In the solitary tract nucleus, this downregulation is delayed. In the prefrontal cortex, downregulation occurs only transiently, leading to a minor receptor upregulation after 4 weeks.

experiments showed that the effects on the receptor numbers were time and brain region dependent. In the LC, α_2-ARs were already downregulated 2 days after the onset of the stress period and stayed low thereafter (Fig. 9). As pointed out earlier, this decrease in binding site numbers may represent agonist-mediated downregulation of α_2-ARs. However, downregulation of the receptor may also be brought about by other factors, e.g., it may be related to steroid hormone-induced changes in gene expression such as the transcription of the renal α_{2B}-AR, which has been reported to be regulated positively by testosterone (Gong *et al.,* 1995). Because subordinate male tree shrews show testicular atrophy and low levels of plasma testosterone, it cannot be excluded that the deficit in testosterone and the accompanying changes in steroid metabolism impair α_2-AR gene transcription (Fischer *et al.,* 1985). Glucocorticoids that are constantly elevated during PSS in tree shrews could also have an influence on α_2-ARs, as it has been shown that adrenalectomy (which reduces plasma glucocorticoid levels down to zero) induced a corticosterone reversible decrease in α_2-AR binding in the paraventricular nucleus of the hypothalamus and an increase in the supraoptic nucleus (Jhanwar-Uniyal and Leibowitz, 1986). Furthermore, *in vitro* experiments indicate that other compounds, such as the second messenger cyclic AMP, also have to be considered as regulatory factors for α_2-AR gene expression *in vivo* (Sakaue and Hoffman, 1991).

As described earlier, α_2-adrenergic autoreceptors are located on noradrenergic neurons where they inhibit noradrenaline release (Aghajanian *et al.,* 1977). The decrease in receptor numbers, which was observed shortly after the onset of PSS in the LC, is likely to lower the capacity for negative feedback regulation of noradrenaline release. Such an impairment of the α_2-AR-mediated negative feedback control mechanism could be an explanation for accelerated noradrenergic activity in the LC system under prolonged stress. Because it is well documented that α_2-AR agonists produce sedation, supposedly being mediated by the LC, the α_2-AR decrease in chronic stress would have an impact on the responsiveness to sedatives, a well-known phenomenon in anesthesia (Kayser *et al.,* 1992; Pertovaara *et al.,* 1994). Under normal conditions, the α_2-AR-mediated inhibition of LC neuron firing and the concomitant reduction in noradrenaline release from axon terminals in the thalamus and cerebral cortex make the cortex less receptive for sensory inputs (Ruffolo *et al.,* 1993). This process could protect the organism from gaining an excess of cortical sensory information and may be enhanced by presynaptic receptor stimulation in the cortex. Stress-promoted downregulation of α_2-ARs would reduce the capacity of this protective process and could be a reason for the well-known stress-related hyperresponsiveness toward sensory stimuli.

Other brain nuclei showed a different time course pattern of α_2-AR regulation. In the solitary tract nucleus, downregulation of [^{3}H]RX821002-binding sites was observed from day 21 of the stress period onward. In the prefrontal cortex, a brain area important for the regulation of mood and behavior, α_2-ARs were transiently downregulated after 10 days and finally upregulated after 28 days of PSS, showing that the noradrenergic system adapts to the stress by counterbalancing its receptor numbers (Flügge *et al.,* 1997c). In line with the idea of a long-term adaptive process is the finding of an increase in binding sites for [^{3}H]UK14,304 detected in the cortex of depressed patients (suicide victims; Gonzalez *et al.,* 1994). However, other studies with depressed patients revealed decreased numbers of cortical α_2-ARs (Andorn, 1991). This contradiction in results may be due to differences in the experimental designs because age and pharmacological treatments could both have an impact on α_2-ARs in the brains of patients, factors whose influences are difficult to avoid in clinical studies but can be controlled readily in animal experiments. In any case, the tree shrew data show that α_2-ARs undergo dynamic changes when the nor/adrenergic system is activated during a prolonged time period such as during chronic stress. These receptor changes correlate with deficits in memory performance, which are also stress–time dependent, and persist after cessation of the stress exposure (Ohl and Fuchs, 1998). In all cases, changes in receptor numbers did not exceed 20% of the total number of α_2-adrenergic-binding sites, which is probably due to the fact that with quantitative receptor autoradiography, one determines not only neuronal α_2-AR binding but also receptors on glial structures.

In one of the brain nuclei, the dorsal motor nucleus of the vagus, PSS not only changed α_2-AR numbers but, in addition, altered affinities for the agonists oxymetazoline and UK14,304 (Flügge, 1996). Because it has been shown *in vitro* that agonist-induced desensitization of α_2-ARs can occur within minutes, it is probable that these changes are mediated by high local concentrations of noradrenaline *in vivo* (Boehm *et al.,* 1995). However, it has been discussed that alterations in α_2-AR affinities *in vivo* may be related to changes in steroid levels, as estradiol modified the thermodynamic parameters of [^{3}H]RX821002 binding in the rat hypothalamus (Karkanias and Etgen, 1995). In this study in the female rat, agonist-binding characteristics indicated that estradiol converts the G-protein-coupled receptor to the uncoupled form. It is therefore possible that the stress-induced decrease in affinity for agonists is due to alterations in central nervous steroid concentrations promoting conformational changes in α_2-AR molecules. The modification of receptor affinity appears not to be directly related to the downregulation (i.e., the decrease in receptor number) as during prolonged stress periods in tree shrews it occurs at different times, revealing the strongest effects on day 10 after the onset of the PSS. Thus, the time course pattern

of alterations in receptor affinity did not match the time course pattern of changes in receptor numbers. Receptor affinities showed a tendency to be decreased during the whole period of PSS.

2. α_2-AR Subtype-Specific Regulation

In the experiments described in the previous paragraphs, radioligand-binding studies were used to quantify receptors in the brain. While this method is suitable for the determination of receptor numbers and affinities in distinct brain nuclei, it does not allow the localization of receptor gene expression in single cells, and there is no reliable distinction among the three α_2-AR subtypes. In contrast, with *in situ* hybridization and subsequent light microscopic inspection of brain sections coated with photo emulsion, one can localize α_2-AR subtype expression on the cellular level (Choo, 1994). To obtain probes for *in situ* hybridization experiments, we cloned a 1.2-kb cDNA fragment of the gene for the α_2-AR subtype A of the tree shrew (Meyer *et al.,* 1997). Using this technique, amounts of mRNA for this receptor were determined semiquantitatively in single noradrenergic neurons. Hybridizing a ^{35}S-labeled riboprobe generated from the cDNA fragment with brain stem sections of the tree shrew, we detected an approximately 25% decrease in α_{2A}-AR expression in the noradrenergic LC neurons of subordinate tree shrews after 3 weeks of PSS. The expression of this α_2-AR subtype in noradrenergic neurons indicates that it represents the receptor that triggers the release of noradrenaline from presynaptic terminals (the autoreceptors). This is in line with previous findings showing α_{2A}-AR expression in the LC of the rat (Nicholas *et al.,* 1993a; Scheinin *et al.,* 1994).

The finding of a stress-induced decrease in α_{2A}-AR-specific mRNA in noradrenergic neurons shows that stress regulates the receptors, at least in part, on the level of transcription. In tree shrews, cells in the lateral reticular and the solitary tract nucleus, in the areas of noradrenergic and adrenergic neurons, were found to reduce α_{2A}-AR expression by approximately 20% (Meyer *et al.,* 1997). In contrast, expression of α_{2A}-AR message in the neurons of the dorsal motor nucleus of the vagus was not reduced by stress. Because these preganglionic motoneurons do not synthesize noradrenaline or adrenaline, their α_{2A}-ARs must be involved in functions other than the regulation of catecholamine release. Receptors expressed in neurons of the dorsal motor nucleus of the vagus are located postsynaptically to terminals of noradrenergic fiber originating in other brain nuclei, e.g., in the LC. α_2-ARs that were downregulated in the dorsal motor nucleus of the vagus in the previous receptor-binding studies therefore represent receptors located on fibers originating from other brain nuclei (Flügge, 1996). The fact that the time course pattern of alterations in [^{3}H]RX821002 binding in the dorsal

motor nucleus of the vagus is the same as in the LC indicates that the receptors in the dorsal motor nucleus of the vagus are located on noradrenergic fibers from the LC.

B. Brain β-ARs under Stress

Not only the α_2-ARs but also β-ARs are affected by the stress-related noradrenergic hyperactivity in the brain, and reduced numbers of these receptors have been suspected to be a biochemical factor underlying the adaptation to stress (Stone and Platt, 1982; Areso and Frazer, 1991). To investigate whether stress effects on the β-AR system might also follow a certain time course pattern and to find out which brain areas are most affected, we quantified numbers of binding sites for the antagonist ^{125}I-iodocyanopindolol (^{125}ICYP) by *in vitro* receptor autoradiography in the brains of male tree shrews submitted to chronic PSS. Because this ligand not only interacts with β-ARs but also with certain serotonin receptors, we first determined its binding characteristics in the tree shrew brain (Flügge *et al.,* 1997a). As expected, ^{125}ICYP revealed a high degree of nonspecific binding, and for a quantitative evaluation of β-ARs, nonspecific sites had to be blocked by 100 μM 5HT. To distinguish between β_1- and β_2-ARs we used the subtype selective ligands CGP20712A, a β_1-AR antagonist, and the β_2-antagonist ICI118.551. The concentrations necessary to block either β_1 or β_2-ARs were determined in competition experiments, meaning that tissue sections were incubated with one concentration of radioligand and varying concentrations of competitors.

The overall pattern of β-AR distribution in the tree shrew brain resembles that of β_1- and β_2-AR mRNA expression in the rat brain (Nicholas *et al.,* 1993b). The number of β_1-ARs in the tree shrew brain is in general very low, with cortical areas revealing slightly higher receptor numbers than other regions (up to 4 fmol/mg). In all brain areas investigated (olfactory region, frontal and parietal cortex, claustrum, hippocampus, pulvinar nucleus, colliculus superior, and cerebellar molecular layer) there are high- and low-affinity β_1-AR sites, with the low-affinity sites representing a larger population than the high-affinity sites (K_i values for CGP20712A varying between 0.6 nM and 67 μM; Flügge *et al.,* 1997a). In the hippocampus, only low-affinity β_1-ARs were detected (K_i 1.3 μM). β-ARs with heterogeneous affinities have been detected before on slide-mounted sections of rat and human brain, and the radioligand ^{125}ICYP is known to label not only cell surface but also intracellular receptors (Saffitz and Liggett, 1992; Arango *et al.,* 1990). Because it has been demonstrated that on stimulation with an agonist, β-ARs are internalized into the cell whereby they undergo conformational changes that convert them into low-affinity binding sites,

it is possible that the large population of low-affinity sites detected in the tree shrew brain represents internalized receptors. Previous immunocytochemical data on the subcellular distribution of β-ARs already indicated a substantial heterogeneity in the conformation of β-ARs in neurons and glia (Aoki and Pickel, 1992b). This high degree in the variation of conformational states appears to be a special feature of β-ARs, which is probably related to the receptor-specific regulation mechanisms that involve intracellular sequestration. In contrast, the α_{2A}-adrenoceptor, which is not supposed to be internalized, also does not show a large heterogeneity in ligand-binding affinities in the brain (Daunt *et al.,* 1997; Flügge, 1996).

High numbers of β_2-ARs are only found in a few brain structures of tree shrews (Flügge *et al.,* 1997a). In the external layer of the olfactory bulb, claustrum, anteroventral thalamic nucleus, and cerebellar molecular layer up to 55 fmol/mg was detected. For this class of β-ARs, high- and low-affinity radioligand-binding sites were also observed, indicating that these sites represent membrane-bound and internalized β_2-ARs. Only the cerebellar molecular layer displays a high percentage of high-affinity β_2-ARs (K_i for 90% of the receptors in this region was 1.8 n*M* in competition experiments with ICI118.551). It is likely that these receptors are associated with glial fibers that cross the white matter of the cerebellar cortex, as immunocytochemical experiments revealed a high coincidence between the distribution of β-ARs and the distribution of Bergmann glial fibers (Flügge *et al.,* 1997a). PSS in tree shrews resulted in dynamic changes in brain β-ARs displaying stress–time-dependent regional alterations in numbers and affinities (Flügge *et al.,* 1997b; see Fig. 10). After 3 weeks of PSS, a decrease in receptor affinity occurred in cortical areas and in the hippocampus, reflecting changes in the conformations of receptor molecules. After 4 weeks, there was a significant decrease in β-adrenergic binding site numbers in cortical and olfactory regions, which coincides with former findings showing a stress-induced downregulation of β-ARs in the rat brain (Stone and Platt, 1982).

However, there have been several investigations on the role of β-ARs in central nervous stress mechanisms revealing apparently contradictory data. While a positive correlation between β-AR downregulation and adaptation to stress appeared to occur in the study mentioned previously, other investigators found that chronic stress induced an increase in β-AR-binding sites in the rat brain (Basso *et al.,* 1993). Furthermore, ^{125}ICYP binding in various brain regions of the rat, which as a consequence of stress were depressed behaviorally, was not different from that in controls except in the hippocampus, where receptors appeared to be upregulated (Pandey *et al.,* 1995). On the basis of such inconsistent findings it had been concluded that β-adrenergic receptor changes depend on the kind of stress and on test parameters (Brannan *et al.,* 1995). Our results from studies in tree

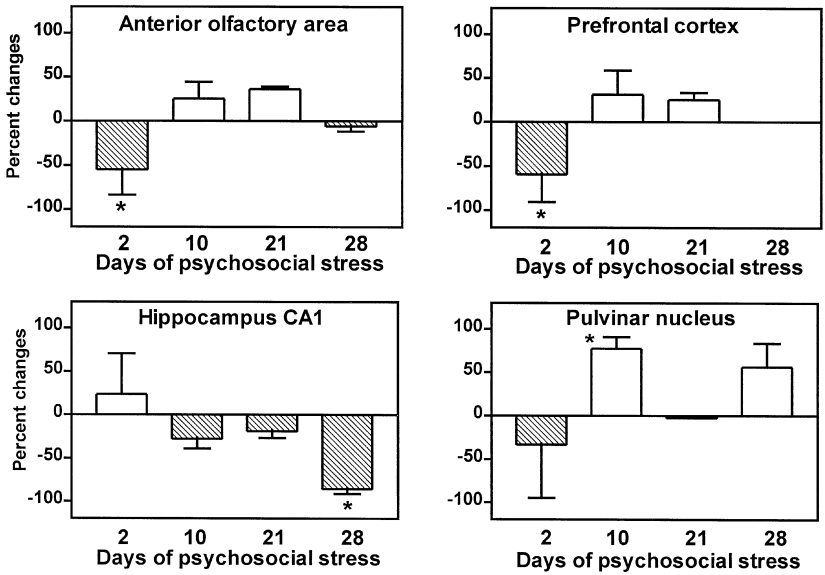

FIG. 10 Changes in β_1-adrenoceptor numbers in the brains of male tree shrews chronically exposed to psychosocial stress for 4 weeks. Receptor numbers were determined by *in vitro* receptor autoradiography with the radioligand ^{125}I-iodocyanopindolol in the presence of serotonin to block nonspecific binding and in the presence of ICI20712A to block binding to β_2-adrenoceptors. Receptor numbers were expressed as percentage of receptor numbers in control animals. Note that in the hippocampus, β_1-adrenoceptors are gradually downregulated during a 4- week period of psychosocial stress, whereas in other brain regions, the regulatory effects are transient.

shrews show that the duration of the stress period is an important parameter and that β_1- and β_2-ARs are regulated independently.

β_1-ARs were downregulated transiently after 2 days of PSS in the prefrontal cortex and the olfactory area and were decreased after 4 weeks of PSS in the parietal cortex and the hippocampus. A transient upregulation of β_1-ARs occurred in the pulviner nucleus after 10 days of PSS. β_2-ARs were downregulated transiently after 2 days of PSS in the prefrontal cortex and upregulated in the pulvinar nucleus after 4 weeks. These results indicate that, on exposure to a stimulus, the β-AR system undergoes rapid desensitization due to its special machinery of intracellular sequestration. Because the intracellular trafficking of β-ARs includes recycling, i.e., reinsertion into the cell membrane, downregulation can be transient, meaning that receptor numbers might return quickly to control levels (Pippig *et al.,* 1995; see also Section III,A,2). In the course of these processes, the receptors

are redistributed between distinct microdomains of the plasma membrane (von Zastrow and Kobilka, 1994). One can assume that these mechanisms are important components of the regulatory apparatus that enables the individual to adapt to situations of recurrent stressful experiences by balancing the number of cellular receptors that are responsive to noradrenergic stimuli.

As described earlier, the neocortex is especially affected by the stress. This part of the brain receives projections from the LC, and high amounts of noradrenaline are released in the cortex, which can lead to receptor downregulation. A similar decrease in receptor numbers has been demonstrated in previous studies showing a negative correlation between β-AR density and the density of noradrenergic projections (Battisti *et al.,* 1989). However, the administration of agents that deplete noradrenaline caused an upregulation of β_1-ARs in the rat brain (Hosoda and Duman, 1993). The assumption that activation of the adrenergic system plays an important part in β-AR expression is sustained by the finding that transgenic mice that overexpress PNMT (the enzyme that catalyzes the biosynthesis of adrenaline) and therefore have high levels of adrenaline show low numbers of β_2-ARs in peripheral organs (Kobayashi *et al.,* 1995). However, the mechanisms of β-AR regulation are complex, involving not only nor/adrenergic systems but possibly also, among other, the serotonergic system. β_1-AR-binding sites were downregulated by chronic antidepressant treatment with serotonin uptake blockers such as desimipramine and imipramine, and by electroconvulsive seizure (Ordway *et al.,* 1988; Hosoda and Duman, 1993). Although the electroconvulsive seizure treatment resulted in a time-dependent downregulation of β_1-AR mRNA, imipramine regulated mRNA levels in a biphasic manner with an increase of β_1-AR gene expression on a 2-week treatment, but a decrease in mRNA levels on a 3-week treatment (Hosoda and Duman, 1993). In any case, the cortical β-AR changes may have considerable implications for neuronal activity as the noradrenergic system modulates the spontaneous firing of cortical neurons and sensory-evoked neuronal discharge patterns, which can influence motivation and behavior (Robbins and Everitt, 1995; Foote and Aston-Jones, 1995).

Because chronic stress has been implicated in the etiology of depression, the findings that stress alters cortical β-ARs have strong implications for human psychiatric diseases. Indeed, in suicide victims, the number of β_1-ARs in the temporal and the frontal cortex was significantly lower than in matched controls (Depaermentier *et al.,* 1993; Little *et al.,* 1993). However, the receptor changes probably not only include neuronal but also glial structures as β-ARs have been detected immunocytochemically on the ultrastructural level in neurons and glia where they may modulate an inwardly rectifying potassium channel (Aoki and Pickel, 1992a; Roy and Sontheimer, 1995). Glial β-AR changes would exert more indirect effects

on cortical activity. The stress effects on β-AR expression in the tree shrew might be in part induced by glucocorticoids that are elevated during chronic stress. Glucocorticoids have been shown to downregulate β_1-ARs (Kiely *et al.,* 1994). In accordance with this view, our results in tree shrews show that stress downregulates β_1-ARs in the hippocampus and cortex, regions known to contain relatively high numbers of glucocorticoid receptors (Jöhren *et al.,* 1994). Why β_2-ARs were also downregulated in these areas, although β_2-adrenergic responsiveness and receptor density can be upregulated by steroids, at least in the lung, needs further clarification (McGraw *et al.,* 1995; Mak *et al.,* 1995). A tissue-specific influence of steroids on gene expression, e.g., via diverse second messenger systems, might underlie this phenomenon. In any case, stress-induced changes in hippocampal β-ARs might affect the functioning of hippocampal pyramidal neurons because it has been shown that the agonist-induced stimulation of spontaneous inhibitory postsynaptic currents in pyramidal cells involves β-adrenergic mechanisms mediated by interneurons (Bergles *et al.,* 1996).

In conclusion, the present findings show that stress alters the numbers and affinities of β-ARs in specific brain regions, indicating an influence of stress on conformation and sequestration of these receptors. Including down- and upregulation of receptors, the effects are transient processes that change in the course of chronic stress. The apparent high flexibility of the β-AR system thus probably enables the brain to adopt quickly to stressful influences.

C. Serotonin$_{1A}$-Receptors under Stress: A Link to Behavior

It has been hypothesized that $5HT_{1A}$-receptor regulation constitutes a neurochemical basis for diverse physiological and behavioral mechanisms that occur during stress. The decrease in hippocampal $5HT_{1A}$-receptors, which was observed in animal models, is considered to be due to the downregulatory influence of plasma glucocorticoids, which are elevated during stress (McKittrick *et al.,* 1995). The assumption that this reduction in hippocampal $5HT_{1A}$-receptor binding coincides with a reduction in the sensitivity of the receptor system was supported by experiments in which the receptor-mediated inhibition of adenylate cyclase activity was tested. Whereas in young rats, cold stress desensitized the second messenger response for only 5 days (inhibition of forskolin-stimulated adenylate cyclase activity was measured), a prolonged desensitization was observed in aged rats (reflected by reduced inhibition of adenylate cyclase activity; Decastro *et al.,* 1996).

In the tree shrew model, $5HT_{1A}$-receptor numbers changed gradually during chronic PSS (Flügge, 1995; see Fig. 11). After 10 days, the number of [^{3}H]8-OH-DPAT-binding sites was reduced in layers V to VI of the

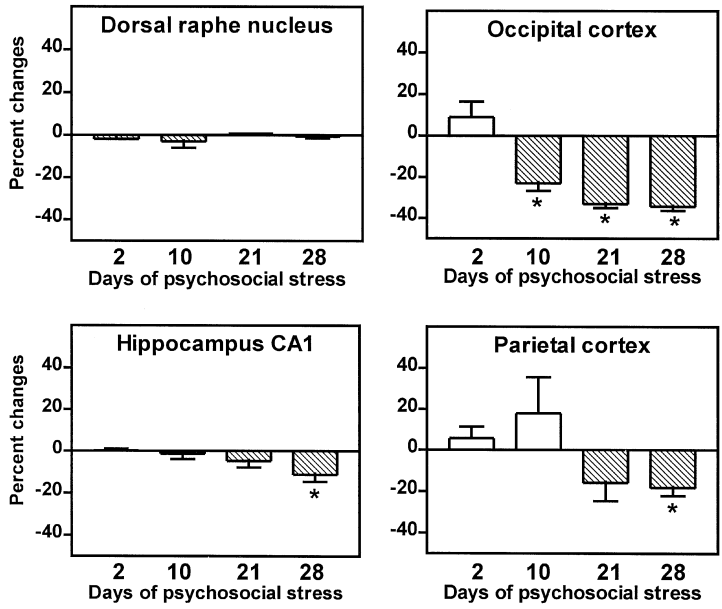

FIG. 11 Changes in $5HT_{1A}$-receptor numbers in the brains of male tree shrews chronically exposed to psychosocial stress for 4 weeks. Receptor numbers were determined by *in vitro* receptor autoradiography with the radioligand [^{3}H]8-OH-DPAT and expressed as percentage of receptor numbers in control animals. Note that $5HT_{1A}$-receptors in the dorsal raphe nucleus, which represent the autoreceptors on the serotonergic neurons, are not changed during a 4-week period of psychosocial stress, whereas in the occipital cortex and the hippocampus, receptors are progressively downregulated.

visual cortex. After 4 weeks, binding sites were also reduced in several other cortical areas and in the hippocampal formation (Flügge, 1995). Responses of the $5HT_{1A}$-receptor system to stress are thus much slower than those of the α_2-AR system. The delayed effect is probably due to the experimental setup in our stress paradigm in which subordinate males are confronted with a dominant conspecific over several days, every morning for only 1 h. The rest of the day, the animals are separated by a wire mesh so that they can see and smell each other, but no physical interaction is possible. Animals are killed in the morning after the last confrontation with the conspecific so that there is no acute stress directly before dissection of the brains, implying the possibility that the receptor system regains its balance during the 1 day "recovery time" after the last social confrontation. The fact that $5HT_{1A}$-receptors in the visual cortex are downregulated after 10 days shows that the system is not able to bring its receptors back to

normal levels within 1 day. In other words, after 4 weeks of repetitive psychosocial stress, the system loses its ability to balance the serotonin receptors within approximately 1 day after a stressful experience. Interestingly, $5HT_{1A}$-receptors in the dorsal raphe nucleus, which represent the autoreceptors on the serotonergic neurons, did not change in the subordinate male tree shrews, even after 4 weeks of daily social confrontations. Only the affinities of the receptors in the dorsal raphe nucleus were slightly reduced, indicating a difference in $5HT_{1A}$-receptor regulation in this nucleus compared to receptors in the hippocampus. It has been shown that there are regional differences in $5HT_{1A}$-receptor regulation. In the dorsal raphe nucleus of rats, $5HT_{1A}$-receptor stimulation inhibited phosphoinositide hydrolysis, whereas in the hippocampus, forskolin-stimulated adenylate cyclase activity was inhibited (Johnson *et al.,* 1997). Electrophysiological data showed that some forms of stress can reduce the $5HT_{1A}$-autoreceptor-dependent inhibitory control of the serotonergic neurons in the dorsal raphe nucleus (Laaris *et al.,* 1997).

While these findings show an influence of stress on $5HT_{1A}$-receptors it has been shown vice versa that $5HT_{1A}$-receptor stimulation itself modulates the adrenocortical response to a stressor. In conscious rats, administration of 8-OH-DAPT at low doses decreased the secretion of corticosteroids, whereas high doses increased plasma corticosterone (Welch *et al.,* 1993). Although one could interpret this as a reflection of the phenomenon that serotonin is a stimulator of the HPA axis, it is possible that the hemodynamic effects of 8-OH-DPAT mentioned previously account for the activation of the HPA axis. However, injections of this agonist into the hypothalamic paraventricular nucleus, the brain nucleus where the ''stress peptide'' corticotropin-releasing factor is synthesized, also activated the sympathoadrenomedullary system and the HPA axis (Korte *et al.,* 1991). Electrophysiological studies have shown that neurons in this nucleus are inhibited by the $5HT_{1A}$-receptor agonist (Saphier and Zhang, 1993). Whether the $5HT_{1A}$-receptor downregulation observed in the just-mentioned brain regions play a role in regulation of the HPA axis response to stress is currently unknown.

Studies in male rats indicated a link between $5HT_{1A}$-receptor modulation and subordination behavior, and in male tree shrews, $5HT_{1A}$-receptors in several brain areas of subordinates were downregulated by chronic psychosocial stress (Blanchard *et al.,* 1993; McKittrick *et al.,* 1995; Flügge, 1995). Because subordination in male tree shrews is accompanied by testicular atrophy and reduced testosterone biosynthesis, it was not clear whether the effects of persistent stress are due to the chronic elevation of cortisol or to low testosterone levels (Fischer *et al.,* 1985). We therefore investigated whether testosterone modulates the effects of stress on $5HT_{1A}$-receptors and whether this might be related to the behavioral changes (Flügge *et al.,* 1998). Male tree shrews were submitted to subordination stress for 28 days,

and during the last 18 days, a group was treated with testosterone. The stress reduced scent-marking activity, self-grooming, and the overall locomotor activity of the animals. Testosterone replacement renormalized scent-marking activity and self-grooming behavior, but overall locomotor activity did not return to baseline levels. Brain $5HT_{1A}$-receptors were renormalized in the hippocampal formation and in the occipital cortex, but not in the ventromedial thalamic nucleus. The correlation between the low expression of $5HT_{1A}$-receptors in this nucleus and the low overall locomotor activity is particularly interesting because the ventromedial thalamic nucleus serves as a link in polyneuronal motor circuits interconnecting the cerebral cortex with the basal ganglia and the cerebellum. The $5HT_{1A}$-receptor might modulate the widespread projections of this nucleus to layer I of the neocortex (Flügge *et al.,* 1998). The thalamus therefore also contributes to the $5HT_{1A}$-receptor-mediated modulation of behavior. However, a causality with the stress-related alterations in behavior is clearly indicated by the fact that the testosterone treatment brings $5HT_{1A}$-receptor numbers in certain brain regions back to baseline levels and, at the same time, also renormalizes distinct behavioral parameters (Flügge *et al.,* 1998).

V. Concluding Remarks

Monoamine receptors on neurons in the central nervous system undergo diverse regulatory processes, among which is agonist-induced phosphorylation leading to changes in the conformation of the receptor molecules and thus to receptor desensitization, intracellular sequestration during which the receptors are internalized into the cell and may later be reinserted into the plasma membrane, and receptor downregulation, i.e., a decrease in the number of receptor molecules. These processes vary among the different types of monoamine receptors. α_2-Adrenoceptors are often downregulated by agonists and β-adrenoceptors are internalized rapidly into the cell when stimulated. Others, such as serotonin_{1A}-receptors, are tightly controlled by steroid hormones. Their expression is reduced by the ''stress hormone'' glucocorticoids, whereas gonadal hormones such as testosterone are able to counterbalance the glucocorticoid effects. Because of these mechanisms, the pattern of monoamine receptors in the brain undergoes dynamic changes when the system is activated and when there are high concentrations of agonists and/or their metabolites present in certain regions of the brain or when the hormonal milieu changes. These alterations in the monoamine receptor systems are considered to be partly responsible for changes in the behavior of the individual.

Stress is a physiological situation accompanied by high activity of the noradrenergic and the serotonergic system in the brain. The hyperactivity of noradrenergic neurons in the locus coeruleus, a group of cells in the hindbrain, leads to high concentrations of noradrenaline in various brain regions. During chronic stress, adrenergic receptors are therefore stimulated repetitively by these agonists, which induce receptor downregulation in distinct brain regions. As shown in male tree shrews, a model for chronic psychosocial stress, α_2-adrenoceptors in the locus coeruleus are already downregulated after 2 days of social stress, whereas in other noradrenergic brain centers, downregulation occurs later and follows a different time course pattern. α_2-Adrenoceptors located on noradrenergic neurons function as autoreceptors that trigger the release of noradrenaline via a negative feedback mechanism. The decrease in their number leads to an impairment of the feedback control, which is probably a reason for the increase in the firing frequency of the respective neurons. β-Adrenoceptors respond in a different way to the stress. Shortly after the onset of a stress period, their affinity changes, which reflects alterations in conformation of the receptor molecules due to intracellular sequestration. In the course of a 4-week period of psychosocial stress with repetitive negative social experiences for the individual, the β-adrenoceptor number can renormalize in several brain regions, which is in contrast to most of the stress-induced α_2-adrenoceptor changes. However, in the hippocampal formation, a brain region known to be involved in the regulation of emotional behavior, β_1-adrenoceptors are gradually downregulated by chronic psychosocial stress.

The effects of social stress on serotonin$_{1A}$-receptors occur later than those on adrenoceptors. Numbers of autoreceptors in the dorsal raphe nucleus that trigger the release of serotonin do not change during psychosocial stress, but serotonin$_{1A}$-receptor numbers in the target regions of the dorsal raphe nucleus display a gradual decrease. Respective data are consistent with the idea that they are downregulated by glucocorticoids, ''stress hormones,'' which are elevated during challenging situations. Stress-mediated changes in the number of these receptors coincide with alterations in behavior of the animals. The stress-induced behavioral pattern and, at the same time, the receptor number can be counterbalanced by gonadal steroids. Thus in male animals, which show a deficit in testosterone during periods of social stress, the number of serotonin$_{1A}$-receptors and, at the same time, the stress behavior can be renormalized by exogenous androgen replacement.

In conclusion, there are dynamic changes in brain monoamine receptors during normal physiological processes that are related to changes in other components of the monoarmine system and to alterations in the hormonal environment of the individual.

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Rhodopsin Trafficking and Its Role in Retinal Dystrophies

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We review the sorting/targeting steps involved in the delivery of rhodopsin to the outer segment compartment of highly polarized photoreceptor cells. The transport of rhodopsin includes (1) the sorting/budding of rhodopsin-containing vesicles at the *trans*-Golgi network, (2) the directional translocation of rhodopsin-bearing vesicles through the inner segment, and (3) the delivery of rhodopsin across the connecting cilium to the outer segment. Several independent lines of evidence suggest that the carboxyl-terminal, cytoplasmic tail of rhodopsin is involved in the post-Golgi trafficking of rhodopsin. Inappropriate subcellular targeting of naturally occurring rhodopsin mutants *in vivo* leads to photoreceptor cell death. Thus, the genes encoding mutations in the cellular components involved in photoreceptor protein transport are likely candidate genes for retinal dystrophies.
KEY WORDS: Rhodopsin, Photoreceptor, Sorting signal, Polarized trafficking, Molecular motors, Retinitis pigmentosa.

I. Introduction

The light-sensitive photoreceptor is a highly polarized and compartmentalized neuron, both in function and in morphology. Rhodopsin, the visual pigment of the rod photoreceptor, is synthesized and processed in the cell body, and it undergoes directional translocation through the inner segment

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to the base of the connecting cilium prior to its further incorporation into the disk membrane of the outer segment, where phototransduction occurs. In vertebrates, the removal of the apex of the outer segment by the adjacent retinal pigment epithelium is balanced by the constant addition of newly synthesized membranes and proteins at the base of the outer segment. The translocation of rhodopsin toward the outer segment is thought to occur on rhodopsin-laden membrane vesicles budded from the *trans*-Golgi network in which proteins destined for different cell domains are sorted. Specific mechanisms must exist to regulate this high-fidelity transport in photoreceptors.

In this review, we begin with a discussion of the polarized cytoskeletal structures of photoreceptors that might be related to the vectorial transport of photoreceptor proteins. We review the pioneering studies that have established the foundation of knowledge about rhodopsin's trafficking routes *in vivo*. These results suggest that multiple sorting decisions and steps are likely to be required for protein targeting in a cell with such complexity. Recent identification and characterization of rhodopsin mutations responsible for some types of retinitis pigmentosa, an inherited, progressive retinal degeneration disease, have led to breakthroughs in unraveling the sorting/targeting signals of rhodopsin. We will then discuss the characterization of the sorting/targeting signals of rhodopsin through studies in cell-free systems as well as in polarized epithelial cells. We describe recent progress in understanding the mechanisms of each step of rhodopsin's transport: from the sorting/budding of rhodopsin-bearing membrane vesicles to the vectorial movement of these vesicles by motor proteins across the inner segment and finally across the connecting cilium. In the final section, we discuss how defects in rhodopsin trafficking could lead to photoreceptor death. Although the answer remains speculative, proteins engaged in photoreceptor trafficking are good candidates for causing retinal degenerative diseases.

II. The Rod Photoreceptor: A Highly Polarized Cell

A. Polarized Morphology

Photoreceptors are phototransducing neurons located in the outermost layer of the neural retina. The photoreceptor cells in mammals are typically about 1–2 μm in diameter and 40 μm in length. In contrast, the photoreceptors in amphibians (such as frogs and salamanders) are about 5 μm in diameter and 55 μm in length, the largest in the animal kingdom. Although photoreceptors vary greatly in size among different species, their structure

is similar in all vertebrates. These neurons are highly polarized in both morphology and function. Each vertebrate photoreceptor can be divided into four histologically distinct compartments (Fig. 1A): (1) the outer segment (OS); (2) the inner segment (IS), (3) the cell body, and (4) the synaptic terminal. The OS is the most distal portion of the photoreceptor and is where the phototransduction cascade occurs. The OS is composed of hundreds of flattened membrane disks stacked in close apposition and enclosed

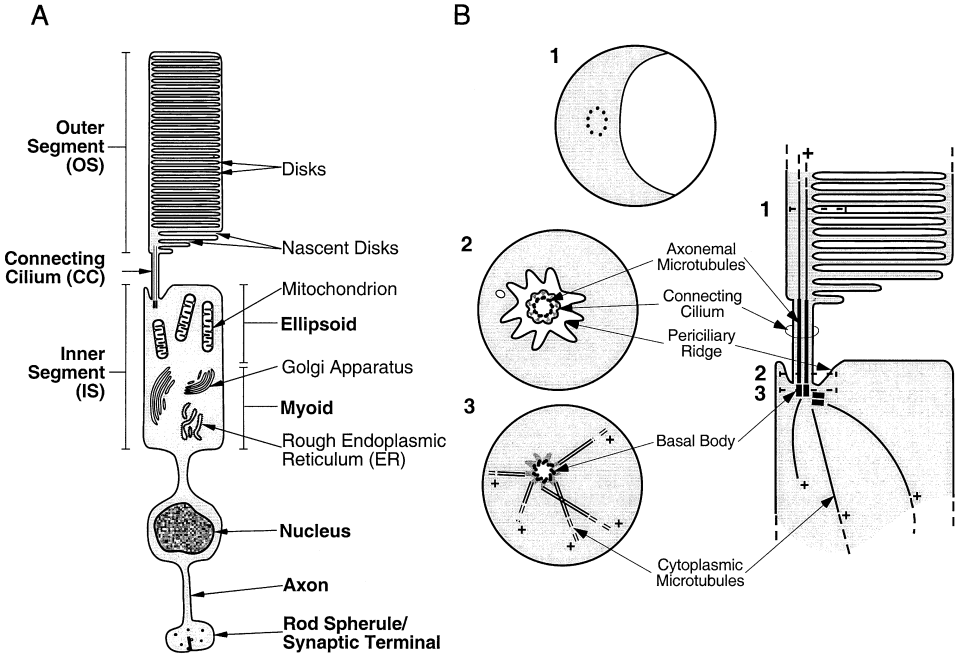


FIG. 1 Photoreceptor anatomy and microtubule organization. (A) The vertebrate rod photoreceptor is highly polarized, both functionally and morphologically. Each rod is divided into several distinct anatomic compartments. The OS contains hundreds of rhodopsin-rich membrane disks stacked within a plasma membrane envelope. The IS is traditionally subdivided into the distal ellipsoid, which is densely packed with mitochondria, and the proximal myoid, which contains most of the cell's biosynthetic machinery such as the ER and the Golgi apparatus. The remainder of the photoreceptor comprises the nucleus, a short axon, and the rod spherule/synaptic terminal. The OS and IS are joined via a nonmotile CC. (B) Schematic of microtubule organization in the rod photoreceptor. There are two populations of microtubules, both of which are organized with uniform polarity (Troutt and Burnside, 1988; Troutt *et al.,* 1990). Axonemal microtubules nucleate from the basal body (section 3) at the distal end of the IS and extend in a 9+0 configuration through the CC (section 2). As they enter the OS, microtubule doublets become singlets (section 1). Cytoplasmic microtubules, on the other hand, are longitudinally aligned with their "minus" ends directed toward the basal bodies and their "plus" ends toward the synaptic terminal.

by a continuous OS plasma membrane. In rod cells the disk membranes are completely sealed, except for those that are in the process of formation at the base of the OS. In contrast, cone cell disk membranes are continuous with the cone OS plasma membrane. The distal region of the IS, called the ellipsoid in nonmammalian vertebrates, is densely packed with mitochondria that are used to support the high metabolic demands of photoreceptors. The proximal IS, termed the myoid in nonmammalian vertebrates, contains essentially all of the cell's biosynthetic machinery, for example, rough endoplasmic reticulum (ER), free ribosomes, and the Golgi apparatus. The cell body contains the nucleus, and the synaptic terminal makes contact with horizontal and bipolar neurons in the inner part of the retina. The synaptic terminal of the rod cell, called the rod spherule, is a small, round synaptic profile with a single synaptic ribbon. The synaptic terminal of the cone cell, called the pedicle, is larger and flatter and contains several ribbons.

The OS and IS are physically connected by a slender stalk called the connecting cilium (CC). About 0.3 μm in diameter in amphibians and 0.17 μm in diameter in most mammals, the CC has a total length of about 0.2–0.5 μm. In cross section, the CC contains nine circularly arranged axonemal microtubule doublets that extend from the basal body at the distal end of the IS and extend into the proximal OS (Fig. 1B); note that the photoreceptor axoneme lacks the central microtubule doublet found in motile cilia. Although microtubules are the most obvious cytoskeletal elements, actin and actin-associated proteins are also found in the CC (see Section II,B,2; Chaitin *et al.,* 1984; Vaughan and Fisher, 1987; Arikawa and Williams, 1989; Chaitin and Coelho, 1992; Williams *et al.,* 1992). From an ultrastructural point of view, the CC is analogous to the transitional zone of motile cilia (Röhlich, 1975; Besharse and Horst, 1990; Horst *et al.,* 1990), whereas the OS is an enlarged and modified extension of the cilium. The strategic position of the CC in photoreceptor cells suggests that it is critical in photoreceptor functions that we will discuss in Sections IV,D and VI,B,2.

Freeze-fracture and high resolution scanning electron microscopy techniques (Andrews, 1982; Peters *et al.,* 1983) have identified a highly organized complex on the apical surface of the frog photoreceptor IS termed the periciliary ridge complex. The periciliary ridge complex is characterized by a deep plasma membrane invagination at the apical membrane of the IS surrounding the proximal end of the CC (Fig. 1B). The lateral wall of the invagination is deeply indented by nine symmetrically arrayed ridges and grooves that extend ~0.4–1 μm in length and 0.3 μm in depth in the frog photoreceptor. The ninefold radial symmetry of the complex and its apparent alignment with the axonemal microtubule doublets (Fig. 1B) suggest that this structure may be somehow related to the ninefold symmetry of the CC axonemal microtubules. Because of the location of the pericili-

ary ridge complex in relation to the IS and OS, and the relatively high density of rhodopsin on the periciliary membrane (Nir and Papermaster, 1983; Peters *et al.,* 1983), it has been proposed that it may function as a collection area for rhodopsin prior to its subsequent translocation to the OS (see Section IV,D; Peters *et al.,* 1983). Consistent with this proposal is the observation of cytoplasmic rhodopsin-containing vesicular profiles in association with the grooves of the complex (Peters *et al.,* 1983; Papermaster *et al.,* 1985, 1986).

B. Polarized Cytoskeletal Organization

The polarity of the photoreceptor with respect to its morphology and protein distribution is paralleled by its high degree of cytoskeletal polarization. The observation that proteins (such as rhodopsin) normally localized to the OS in mature photoreceptors are distributed on the entire plasma membrane of the IS in postnatal rats prior to OS formation suggests that the mature cell cytoskeleton and CC may be involved in the establishment and maintenance of photoreceptor polarity (Nir *et al.,* 1984).

1. Axonemal and Cytoplasmic Microtubule Organization

In eukaryotic cells a specialized organelle called the microtubule organizing center (MTOC) or centrosome is responsible for maintaining microtubules in a polarized array in interphase cells. The ''fast-growing'' and ''slow growing'' ends of microtubules are respectively referred to as the ''plus'' and the ''minus'' ends (Allen and Borisy, 1974; Dentler *et al.,* 1974). In most cell types, such as fibroblasts, the MTOC is located near the nucleus and the microtubule array radiates toward the cell periphery (Euteneuer and McIntosh, 1981; Soltys and Borisy, 1985). However, no centrioles or other morphologically identifiable MTOCs have been observed in the perinuclear region of photoreceptors (Kinney and Fisher, 1978; Warren and Burnside, 1978). Instead, like other postmitotic ciliated cells, the major MTOC in the photoreceptor is the basal body situated at the base of the CC (Troutt *et al.,* 1990). The basal body itself consists of two centrioles composed of nine triplet microtubules surrounded by electron-dense amorphous pericentriolar material (Fig. 1B). In addition to morphological evidence that the basal body is a MTOC, two centrosomal markers—centrin (Wolfrum, 1995) and γ-tubulin (Muresan *et al.,* 1993)—are found in the basal bodies as well as in the CC by immunocytochemistry.

Unlike most eukaryotic cells, photoreceptors possess two distinct populations of microtubules: axonemal microtubules and cytoplasmic microtubules. The axonemal microtubules arise as doublets from the basal body

and run through the CC in a modified $9 \times 2 + 0$ configuration that is characteristic of nonmotile sensory cilia (Fig. 1B). Note the lack of typical dynein arms; however, Y-shaped cross-links between the doublets and the plasma membrane, as well as nexin links between adjacent doublets, can be seen by electron microscopy and could potentially contribute to the maintenance of an IS–OS diffusion barrier (see Section IV,D; Richardson, 1969; Röhlich, 1975; Peters *et al.,* 1983; Chaitin and Burnside, 1989). These axonemal microtubules are uniformly oriented with their ''minus'' ends at the basal body and their ''plus'' ends directed toward the OS (Troutt and Burnside, 1988; Troutt *et al.,* 1990). The doublet microtubules become singlet ($9 \times 1 + 0$) in the OS and finally disappear at a considerable distance distal to the CC (Fig. 1B; Kaplan *et al.,* 1987; Roof *et al.,* 1991).

In the inner part of the photoreceptor, singlet cytoplasmic microtubules run through the IS, the cell body, and the axon. Cold-induced microtubule disassembly and reassembly experiments conducted in fish photoreceptors suggest that these cytoplasmic microtubules are nucleated at the basal body (Troutt *et al.,* 1990). The polarity of these microtubules has also been determined by electron microscopic analysis using microtubule ''hooking'' methods: virtually all are oriented with their ''minus'' ends directed toward the basal body and their ''plus'' ends directed toward the axon terminal (Fig. 1B; Troutt and Burnside, 1988; Troutt *et al.,* 1990). The organization of microtubules is similar in both rods and cones; however, many more microtubules are found in cones (Troutt and Burnside, 1988). Although these detailed studies of microtubule polarity have so far only been performed in teleost photoreceptors, it is likely that mammalian and amphibian photoreceptors share a similar microtubule organization. It is worth noting that the layout of microtubules in photoreceptors is different from that in conventional neurons. In neuronal axons, the microtubules are polarized with their ''minus'' ends toward the cell body and their ''plus'' ends toward the terminals (Heidemann *et al.,* 1981), whereas in dendrites the microtubules are of mixed polarity (Baas *et al.,* 1988).

In fish, amphibians, and birds, both rods and cones undergo extensive length changes in response to changes in lighting conditions (Burnside and Nagle, 1983). Cones contract and elongate in response to the onset of light and darkness, respectively. Rods respond to light and darkness in the opposite fashion. Elongation and contraction are controlled by changes in the length of the myoid region. Studies using cytoskeletal-disrupting agents have shown that cone myoid elongation is dependent on intact microtubules and contraction is dependent on intact actin (Burnside, 1976; Warren and Burnside, 1978). However, in rods, both elongation and contraction are actin-dependent; microtubule-disrupting drugs show no effect on rod length changes (O'Connor and Burnside, 1981, 1982).

2. Actin Filament Organization

The actin filaments in photoreceptors are also specifically organized. Initial studies using immunoelectron microscopy as well as fluorescent phalloidin labeling of F-actin have revealed that F-actin present at the base of the OS, where nascent disks are formed (Chaitin *et al.,* 1984; Chaitin and Bok, 1986; Del Priore *et al.,* 1987; Vaughan and Fisher, 1987). Another approach has employed electron microscopy to directly visualize actin filaments with or without prior decoration by myosin subfragment-1 (Arikawa and Williams, 1989; Chaitin and Burnside, 1989). This latter method is useful in determining the polarity of actin filaments: the ''barbed'' ends are faster growing and the ''pointed'' ends are slower growing. At the base of the OS, short filaments project radially from the center of the ciliary axoneme in all directions, pass between ciliary microtubule doublets, and attach to the plasma membrane with their ''barbed'' ends. Some of these actin filaments extend into liplike plasma membrane protrusions at the base of the OS that may represent an early stage of nascent OS disks, but are absent from distal nascent disks. The location of these filaments and the observation that microfilament disruption by cytochalasin D causes an aberrant overgrowth of nascent open disks in *Xenopus* photoreceptors (Williams *et al.,* 1988) indicate that actin may be involved in disk morphogenesis (discussed in further detail in Section IV,E). Actin disruption does not appear to qualitatively inhibit either the incorporation of membrane or of newly synthesized rhodopsin into aberrant disks (Williams *et al.,* 1988). In contrast to the shorter radially oriented filaments, a few longer actin filaments are found to extend longitudinally along the axoneme in both directions (distally and proximally) away from the short radial filaments. All of these longer filaments have their ''pointed'' ends facing the short filaments (Arikawa and Williams, 1989). Although the function of this population of longitudinal filaments is unclear, it can be speculated that they may be involved in protein transport across the CC (see Section VI,B,2,c).

In the IS of vertebrate photoreceptors, fluorescent phalloidin labeling of F-actin and electron microscopy demonstrate the presence of longitudinally oriented actin filament bundles just under the IS plasma membrane that extend toward the OS (Nagle *et al.,* 1986; Del Priore *et al.,* 1987; Vaughan and Fisher, 1987). These actin filaments may be involved in the regulation of myoid length in response to light in fish, amphibian, and bird photoreceptors as discussed in Section II,B,1. In addition, a ringlike staining pattern can be seen in photoreceptors at level of the outer limiting membrane of the retina, corresponding to a zonula adherens-like junction between photoreceptors and Müller glial cells (Vaughan and Fisher, 1987; Williams *et al.,* 1990).

3. Other Cytoskeletal Structures

The distribution of intermediate filaments in photoreceptors has not been intensively studied. In teleost cones, intermediate filaments are relatively sparse in the IS but are abundant in the perinuclear region and axon (Nagle *et al.,* 1986).

Like other types of ciliated cells, each photoreceptor also contains a single cross-striated fibrous structure termed the ciliary rootlet. A ciliary rootlet extends continuously from the proximal end of each basal body through the entire IS and ends at the synaptic terminal (Olson and Rattner, 1975). This structure is not recognized by actin or tubulin antibodies (Chaitin and Bok, 1986; U. Wolfrum, personal communication, 1998) and its molecular components remain unknown. In guinea pig photoreceptors, the ciliary rootlet is flanked by membranous saccules (Spira and Milman, 1979). In rat photoreceptors, ciliary rootlets are associated with ER and mitochondrial membranes, suggesting that they may have a role in organelle organization and/or motility (Wolfrum, 1992).

C. Polarized Outer Segment Localization of Rhodopsin

Rhodopsin is the light-transducing visual pigment of rod photoreceptors. Each rhodospin molecule is composed of the apoprotein opsin and the chromophore 11-*cis*-retinal (for reviews, see Nathans, 1992; Sakmar, 1998). For the sake of convenience, we will use the term ''rhodopsin'' throughout this review to refer to both opsin and rhodopsin. Hydropathy modeling and topologic studies have shown that rhodopsin is a seven-transmembrane protein with its carboxyl terminus facing the cytoplasmic (interdiscal) side and its amino terminus facing the extracellular (intradiscal) side of the lipid membrane (Fig. 3). Dual palmitoylation sites located at amino acid residues 322 and 323 are conserved among all mammalian rhodopsins. The retinal group is Schiff-base conjugated to lysine residue 296 of rhodopsin, which is located in the middle of the seventh transmembrane helix.

The OS plasma membrane and disk membrane are extremely rich in rhodopsin. It has been estimated that 85–90% of the total OS protein and over 95% of the total disk membrane protein is rhodopsin (Hall *et al.,* 1968; Basinger *et al.,* 1976a; Krebs and Kühn, 1977). This corresponds to approximately 5×10^7 molecules of rhodopsin in each OS (Knowles and Dartnall, 1977) and 2×10^4 molecules per square micrometer of disk membrane (Corless *et al.,* 1976). Despite the extremely high density of rhodopsin in the OS, immunoelectron microscopic studies reveal that there is comparatively very little rhodopsin on the plasma membrane surrounding the IS, cell body, and synaptic terminal, with the exception of the periciliary

ridge complex (Nir and Papermaster, 1983). This observation is corroborated by a variety of other techniques (Defoe and Besharse, 1985; Spencer *et al.,* 1988; Sung *et al.,* 1994; Besharse and Wetzel, 1995). The rod Golgi apparatus, though, typically contains high concentrations of rhodopsin, consistent with its high rate of synthesis (Papermaster *et al.,* 1978; Röhlich *et al.,* 1989).

The high concentration of rhodopsin in the rod OS and its rapid turnover (see Section III) may represent the most extreme example of polarized protein distribution in all systems examined so far. Moreover, since the OS lacks any biosynthetic machinery (Young, 1968), all protein and lipid components of the OS must first be synthesized in the proximal IS and subsequently delivered to the OS. It would be thus reasonable to expect that the cell has an active transport pathway to concentrate rhodopsin in this compartment. In later sections we will discuss the evidence that rhodopsin transport is directed, at least in part, by the recognition of sorting/targeting signal(s) on the rhodopsin polypeptide by one or more cellular factors.

III. Rapid Turnover of Rod Disk Membranes

A remarkable feature of the photoreceptor is that the OS and its components are continuously and rapidly renewed throughout the lifetime of the animal. The tip of the OS is reguarly shed and phagocytosed by the adjacent retinal pigment epithelium (RPE) cells, while at the base of the OS new disks are assembled from new proteins and lipids synthesized in the IS. One intriguing piece of evidence that this turnover is physiologically important comes from the Royal College of Surgeons (RCS) rat. These animals have an inherited defect in RPE phagocytosis of shed OS membranes and also suffer from retinal degeneration (Edwards and Szamier, 1977; LaVail, 1983). Thus, it appears that coordination between OS breakdown and synthesis is critical to the health of photoreceptors.

The process of OS membrane renewal was first directly demonstrated by tissue autoradiography. In this technique, retinas previously radiolabeled by injection (intravascular, intraperitoneal, or intraocular) of ^{3}H-labeled amino acids are fixed, sectioned, mounted, coated with photographic emulsion, and developed (Droz, 1963; Bok, 1982). The sections are viewed by light or electron microscopy, and the positions of the silver grains indicate the positions of newly synthesized proteins. In frog and rodent photoreceptors, silver grains first appear in the IS (but not in the OS) within minutes of injection, and by 1 hr grains can be seen over the Golgi apparatus (Droz, 1963; Young, 1967; Young and Droz, 1968; Hall *et al.,* 1969; Bok, 1982). Within hours of injection, silver grains appear over the distal IS and at the

base of the OS. These observations demonstrate that the biosynthesis of OS proteins in the photoreceptor cell takes place solely in the IS. Biochemical analysis reveals that about 85% of the OS-incorporated radiolabel is associated with rhodopsin (Hall *et al.,* 1968), indicating that the progression of silver grains to rod OSs largely represents the movement of newly synthesized rhodopsin molecules to the OS. Moreover, the labeling intensity of these bands of silver grains and the specific activity of the radiolabeled rhodopsin extracted from isolated OSs do not measurably decrease over time, suggesting that little or no turnover occurs once proteins become incorporated into rod OS disk membranes (Hall *et al.,* 1968). An *in vitro* system in which explanted retinas are metabolically labeled in culture gives results fully consistent with the *in vivo* data (Basinger and Hall, 1973; Basinger and Hoffman, 1982).

One key observation in these tissue autoradiography experiments is that with increasing chase times, ^{3}H-radiolabeled rod disks become progressively displaced toward the OS distal tip, suggesting that the pulse-labeled disks are being displaced by newly synthesized, unlabeled disks at the base of the OS. On reaching the tip of the OS, radiolabeled disks are shed in packets, which are then phagocytosed, proteolytically digested, and ultimately eliminated by the adjacent RPE (Young and Bok, 1969). The OS turns over approximately every 10 days in mammals and every 6 weeks in frogs (Young, 1967). The extraordinary magnitude of this turnover rate can be better appreciated from stereological measurements of disk surface area and photoreceptor numbers. Given the estimate that a rat retina contains about 770 cm^2 of disk membrane, then about 77 cm^2 of disk membrane must be shed and synthesized anew each day (Mayhew and Astle, 1997). This is equivalent to over 50 times the outer surface area of the eyeball. Although the phenomenon of rapid OS renewal has been thoroughly described, why it occurs at such a rapid rate is less clear. There is some evidence to suggest that ''old'' distal disks have different properties from ''new'' basal disks. These properties include the degree of rhodopsin bleaching and rate of regeneration (Williams and Penn, 1985; Makino *et al.,* 1990), extent of membrane protein phosphorylation on exposure to light (Shichi and Williams, 1979; Boesze-Battaglia *et al.,* 1996), single photon responsiveness (Schnapf, 1983), and possibly cholesterol content (Andrews and Cohen, 1979; Caldwell and McLaughlin, 1985; Boesze-Battaglia *et al.,* 1989). It is conceivable, therefore, that an as-yet undefined ''aging'' process occurs in OS disks that necessitates their rapid replacement.

A. Regulation of Disk Shedding

The OS disk formation and shedding processes are not continuous and in fact follow a cyclic rhythm. This rhythm appears to be controlled to varying

degrees in different species by apparently distinct mechanisms. In all vertebrate retinas studied to date, a burst of disk shedding occurs synchronously within 1–2 hr after light onset (Basinger *et al.,* 1976b; LaVail, 1976; Besharse *et al.,* 1977a). Evidence shows that disk shedding in *Rana pipiens* frogs is controlled primarily by a diurnal (light-stimulated) rhythm (Basinger *et al.,* 1976b; Besharse *et al.,* 1977b), whereas disk shedding in *Xenopus laevis* frogs and mammalian rods appears to be controlled primarily by a light-entrained circadian oscillator (La Vail, 1976, 1980; Besharse *et al.,* 1977a; Besharse and Hollyfield, 1979). *Xenopus laevis* frogs kept in continuous darkness for 6 days show reduced shedding and significantly elongated OSs. On light exposure a large burst of synchronous disk shedding is observed. In contrast, disk shedding in rats follows a cyclic free-running rhythm of slightly more than 24 hr for up to 12 days of continuous darkness and the circadian rhythm can be re-entrained to a phase-shifted light cycle (LaVail, 1976, 1980; Besharse *et al.,* 1977a; Besharse and Hollyfield, 1979). The molecular mechanisms by which light or circadian rhythms regulate OS turnover are largely unknown.

B. Regulation of Disk Synthesis

It is reasonable to imagine that variations in the rate of disk shedding are balanced by regulating new disk formation. In *R. pipiens,* the rate of radiolabeled disk displacement is significantly decreased in animals kept in constant darkness. Exposure of frogs maintained in constant darkness to 24 hours of continuous light results in a dramatic burst of disk synthesis (Besharse *et al.,* 1977a,b), indicating that in *R. pipiens,* disk synthesis, like disk shedding, is stimulated directly by light. Likewise, rhodopsin protein synthesis increases shortly after light onset in this species (Matsumoto and Bok, 1984). Immunogold electron microscopic labeling of rhodopsin also reveals a diurnal pattern in *R. pipiens* photoreceptors: rhodopsin immunoreactivity on ER and Golgi membranes increases throughout the day, then decreases during the dark phase, reaching a nadir by light onset (Bird *et al.,* 1988).

The relationship between light and disk synthesis is a more complex issue in other species. In *X. laevis,* the rate of radiolabeled disk displacement is higher during the first 8 hr of the light cycle than during the remaining 16 hr (Besharse *et al.,* 1977b). Measurement of the rate of disk assembly in *X. laevis* by counting the number of open nascent disks at the base of rod OSs indicates that the rate of disk assembly is stimulated by light (Hollyfield *et al.,* 1982). However, no variation in the rate of ^{3}H-labeled amino acid incorporation into rhodopsin in *X. laevis* frogs can be found under various lighting conditions (Hollyfield and Anderson, 1982; Hollyfield

et al., 1982). The discrepancy between rhodopsin synthesis and disk assembly in relation to light in *X. laevis* has been interpreted to suggest that, in these animals, light stimulates the assembly of new OS disks from previously synthesized protein.

In mice, no detectable differences in the magnitude of radiolabeled disk displacement and the rate of disk synthesis are observed after several days of constant light or darkness compared to cyclic light controls (Besharse and Hollyfield, 1979). This result indicates that disk synthesis in mice does not appear to follow a diurnal rhythm. However, the limited time resolution of the experiments cannot rule out the possibility that the rate of disk addition in mice follows a circadian rhythm.

IV. Rhodopsin Trafficking at the Subcellular Level in Normal Rod Photoreceptors

Once it became known that the rod OS was in a state of continuous turnover, attention turned to determining the subcellular routes by which components of the OS, such as rhodopsin, travel through the IS and on the OS. In this section, we describe the experiments that have established our current state of knowledge about the subcellular itinerary of newly synthesized rhodopsin molecules within the photoreceptor.

A. Synthesis and Processing of Rhodopsin in the Rough Endoplasmic Reticulum and Golgi Apparatus

As expected for an integral membrane protein, the rhodopsin polypeptide is synthesized on membrane-associated ribosomes and cotranslationally inserted into the ER membrane in the proximal IS (Goldman and Blobel, 1981). Here and during its subsequent passage through the Golgi stacks, rhodopsin undergoes N-glycosylation and oligosaccharide modification at residues Asn-2 and Asn-15 (for review, see O'Brien, 1982). Passage of newly synthesized rhodopsin through the Golgi apparatus is required for its transport to the OS, as indicated by the fact that monensin, an ionophore that disrupts Golgi organization, blocks transport of rhodopsin to the OS (Matheke *et al.,* 1984; Matheke and Holtzman, 1984).

The possibility that photoreceptors have more than one Golgi apparatus for proteins targeted to different compartments was considered at one time. However, only a single Golgi apparatus is visualized in the photoreceptor by electron microscopy when Golgi membranes are labeled by heavy-metal impregnation or by detection of enzymatic Golgi markers such as thiamine

pyrophosphatase or acid phosphatase (Mercurio and Holtzman, 1982; Matheke and Holtzman, 1984; Schmied and Holtzman, 1989).

B. Sorting of Rhodopsin into Post-Golgi Vesicles

It has been demonstrated in several cell types that proteins in the secretory pathway destined for different membrane domains are sorted into distinct transport vesicles during transit through the *trans*-Golgi network (TGN) (for review, see Wandinger-Ness *et al.,* 1990). This also appears to be true for photoreceptors. Double-label immunoelectron microscopy shows that both rhodopsin and synaptophysin, molecules destined for the opposite ends of the photoreceptor, can be found in the same TGN in photoreceptors, suggesting that the sorting of rhodopsin away from synaptic proteins occurs on or after exit from the TGN (Schmied and Holtzman, 1989).

Biochemical analysis of subcellular fractions isolated from frog retinas that had been pulse-labeled with [^{35}S]methionine and chased for varying lengths of time confirms that polarized sorting of rhodopsin occurs at the photoreceptor TGN. In these studies, labeled rhodopsin is delivered into a fraction containing homogeneous vesicles that resemble typical post-Golgi vesicles in density, size, and morphology (Deretic and Papermaster, 1991). These vesicles also contain the OS proteins transducin and cGMP phosphodiesterase, but do not contain synaptophysin or Na^+K^+-ATPase, markers for the synaptic terminal and the IS plasma membrane, respectively. These data imply that rhodopsin is sorted in a polarized manner at or prior to post-Golgi vesicle formation.

C. Vectorial Movement of Rhodopsin-Bearing Post-Golgi Vesicles to the Periciliary Region

The pioneering autoradiographic studies mentioned in Section III provided the first suggestion that newly radiolabeled proteins (consisting mainly of rhodopsin) travel vectorially toward the OS from the perinuclear region (Young, 1968; Young and Droz, 1968; Hall *et al.,* 1969). Radiolabeled proteins accumulate briefly at the base of the CC before finally reaching the base of the OS. Interestingly, radiolabel is not seen at high density over the mitochondria-rich ellipsoid region at any time point, suggesting that the transit of protein through this region is fairly rapid. Very little radiolabel is observed over the lateral plasma membrane of the IS.

Parallel studies using tissue autoradiography and immunoelectron microscopy in frog retinas fixed 2 hr after the injection of ^{3}H-labeled amino acids have revealed that both silver grain radiolabel and rhodopsin immuno-

labeling associate with small vesicular membrane profiles in the distal region of the IS (Papermaster *et al.,* 1985, 1986). These vesicles are often clustered near the periciliary ridge complex at the base of the CC, and vesicles undergoing fusion with the periciliary membrane are occasionally observed. It has been calculated, using a hypothetical source-grain distribution model, that over 30% of the IS radiolabel is associated with these membranous vesicles at 2 hr postinjection. Moreover, similar immunocytochemical localization of rhodopsin to vesicles in the ellipsoid and periciliary regions has been made in freeze-fractured frog retinas (Defoe and Besharse, 1985). Collectively, these data argue strongly that newly synthesized rhodopsin is vectorially carried to the periciliary region in small post-Golgi vesicles. The periciliary ridge complex is likely to represent the destination and preferred site of fusion for these vesicles (Fig. 5). Note that rhodopsin-bearing vesicular–tubular membrane profiles also exist in the IS of mammalian retinas, albeit at lower density than in frog photoreceptors (Wolfrum and Schmitt, 1999).

D. Rhodopsin Transport from Inner to Outer Segments

As discussed above, the itinerary of rhodopsin transport across the IS has been fairly well described. However, the process by which rhodopsin migrates from the apical surface of the IS to the base of the OS, where disk formation occurs, is less clear. There are currently two models of how this transport is accomplished.

The first model proposes that rhodopsin reaches the base of the OS via the CC (Fig. 2A). The CC is the only continuous structure between the IS and OS, and newly synthesized radiolabeled proteins are transiently associated with the CC in autoradiographic experiments (Young, 1968). Additionally, intramembranous particles with a size profile similar to those in disk membranes have been observed on the P-face of the CC plasma membrane by freeze-fracture studies (Besharse and Pfenninger, 1980; Besharse *et al.,* 1985; Miyaguchi and Hashimoto, 1992). Note that since no intracellular membranous structures have ever been reported within the CC, in this model rhodopsin would be transported within the plasma membrane of the CC. However, a major problem with this hypothesis has been the lack of significant rhodopsin labeling on the CC by immunoelectron microscopy (Nir and Papermaster, 1983; Nir *et al.,* 1984; Besharse *et al.,* 1985; Besharse and Wetzel, 1995). Recently, though, a highly sensitive technique using silver-enhanced immunogold postembedding labeling on sodium periodate-etched retinal sections has demonstrated significant levels of specific rhodopsin labeling on the CC plasma membrane of rodent photoreceptors (Fig. 2A, Wolfrum and Schmitt, 1998). In contrast, very little

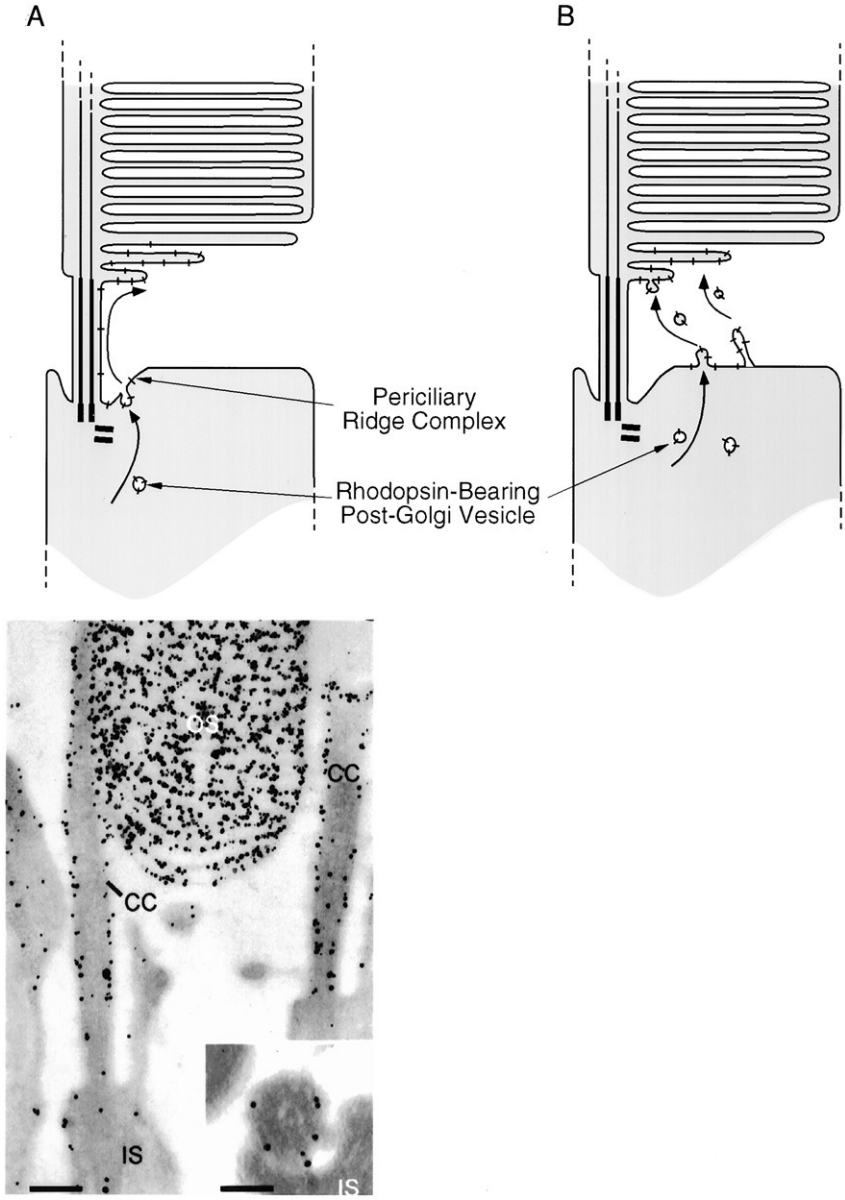


FIG. 2 Schematic diagrams illustrating two different models of rhodopsin passage from the IS to OS. (A) First model: Rhodopsin travels to the OS through the plasma membrane of the CC. Significant levels of silver-enhanced immunogold labeling of rhodopsin can be found on the CC plasma membrane in both a longitudinal section and a cross section (inset) of a rat rod photoreceptor. Note that the OS disk membranes are also heavily labeled. Bar, 250 nm; 150 nm in inset. (Courtesy U. Wolfrum.) (B) Second model: Rhodopsin travels to the OS in extracellular vesicles budded from the IS apical membrane or in transient membrane "bridges" between the IS and OS.

rhodopsin labeling is seen in the center of the ciliary axoneme, supporting the early prediction that rhodopsin travels to the OS via the membrane of the CC.

The second model proposes that rhodopsin is transported to the OS by extracellular vesicles or by transient membrane ''bridges'' between the IS and OS (Fig. 2B). This hypothesis stems from the observation of rhodopsin labeling on distinct processes extending between the IS and OS using immunoelectron microscopy in isolated frog retinas fixed after 1 hr of light exposure in culture (Besharse and Wetzel, 1995). Such intrasegmental connections have been reported in the past (Richardson, 1969), but the possibility that these connections may be artifacts of retinal dissection has not been excluded (Townes-Anderson, 1995). Alternatively, this mechanism may only exist under specific conditions such as during light-stimulated rapid disk assembly (see Section III, B).

Rhodopsin displays very high lateral mobility in OS disk membranes (Liebman and Entine, 1974; Poo and Cone, 1974). Thus, in either model, a diffusion barrier is likely to exist that prevents the back-diffusion of significant amounts of rhodopsin from the OS plasma membrane to the IS via the CC (Spencer *et al.,* 1988). This hypothesis is supported by the observation that when single rods are isolated by mechanical dissociation of retinas, the plasma membranes of the IS and OS frequently fuse with each other; in such fused cells, rhodopsin is found to have redistributed throughout the entire cell's plasma membrane (Spencer *et al.,* 1988). However, the nature and mechanism of this diffusion barrier are unclear.

E. Outer Segment Disk Formation

Newly synthesized proteins and membrane that arrive at the base of the OS are incorporated into newly forming disks. Although it was once believed that disk formation occurred by a process of plasma membrane *invagination,* it now seems more likely that new disks actually arise by plasma membrane *evagination* followed by disk rim formation (Steinberg *et al.,* 1980; Corless and Fetter, 1987). As discussed in Section II,B,2, actin filaments appear to play a role in initiating membrane evagination, possibly by an actin–myosin contractile mechanism, as myosin II has also been detected by immunoelectron microscopy at the base of the OS (Williams, 1991; Chaitin and Coelho, 1992; Williams *et al.,* 1992). The second step of rim formation is thought to be mediated, at least in part, by two disk rim proteins called peripherin/*rds* and rom-1 (Arikawa *et al.,* 1992; Bascom *et al.,* 1992).

Whether rhodopsin is also directly involved in disk formation is somewhat unclear. Mouse rods lacking both rhodopsin alleles fail to develop OSs and

eventually die (Humphries *et al.,* 1997; Lem *et al.,* 1999). However, the failure of OS development in the absence of rhodopsin might not be due to a specific requirement for rhodopsin in disk formation per se, but instead to a requirement for rhodopsin in directing the vectorial trafficking of post-Golgi vesicles carrying OS-destined lipids and proteins, some of which may be specifically involved in OS and/or disk morphogenesis (see Section VI,C).

Another line of evidence that is suggestive of a role for rhodopsin in disk formation is based on studies using tunicamycin, an inhibitor of N-linked oligosaccharide biosynthesis. Tunicamycin treatment of isolated *Xenopus* retinas results in the production of nonglycosylated rhodopsin that is apparently normally transported to the base of the OS; however, normal disks do not form, and the space between the IS and OS becomes filled with extracellular vesicles that seem to originate from disorganized basal OS membranes (Fliesler *et al.,* 1985; Defoe *et al.,* 1986; Ulshafer *et al.,* 1986). It is difficult to tell, however, whether this phenomenon is due specifically to the nonglycosylation of rhodopsin or to the nonglycosylation of another protein required for OS formation. Moreover, disk membrane morphogenesis is unaffected by treatment with castanospermine, an inhibitor of N-linked oligosaccharide trimming which results in the synthesis of hyperglycosylated proteins (Fliesler *et al.,* 1986).

A more specific piece of evidence implicating rhodopsin in disk generation is the observation that transgenic mouse photoreceptors expressing a mutant rhodopsin containing three N-terminal mutations (V20G, P23H, and P27L) have disorganized basal OS disk membranes (Liu *et al.,* 1997a). However, this disorganization appears to be confined to the basal OS, as distal mature OS disks are well organized.

V. Rhodopsin Trafficking in Pathological Conditions

Growing evidence suggests that some hereditary diseases result from defective intracellular protein transport. Examples include the failure of proper transport of low density lipoprotein (LDL) receptor in some types of familial hypercholesterolemia (for review, see Hobbs *et al.,* 1990) and the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (Cheng *et al.,* 1990). Knowledge about rhodopsin trafficking under normal physiological conditions has also been greatly advanced through the identification of naturally occurring rhodopsin mutants and observations about the abnormal processing and intracellular distribution of these mutant rhodopsins *in vivo.*

Retinitis pigmentosa (RP) is one of the most common hereditary retinal dystrophies and affects approximately 1 in 4000 people in all populations

examined (Bundey and Crews, 1984; Bunker *et al.,* 1984). RP typically begins with the loss of rod cell function, causing night blindness and loss of peripheral vision. Central vision, mediated by foveal cone cells, is also affected in the later course of the disease. The majority of patients are legally blind by the age of 60 due to nearly complete photoreceptor degeneration (Berson, 1996). RP is genetically heterogeneous: it can be inherited in X-linked, autosomal recessive, and autosomal dominant forms as well as in a rare digenic mode of inheritance (Kajiwara *et al.,* 1994). Moreover, further genetic heterogeneity exists within each inheritance type. To date, mutations in 10 different genes have been found in patients with RP, and another 17 loci have been linked to different types of RP (RetNet Web site as of 2/25/99; http://www.sph.uth.tmc.edu/retnet).

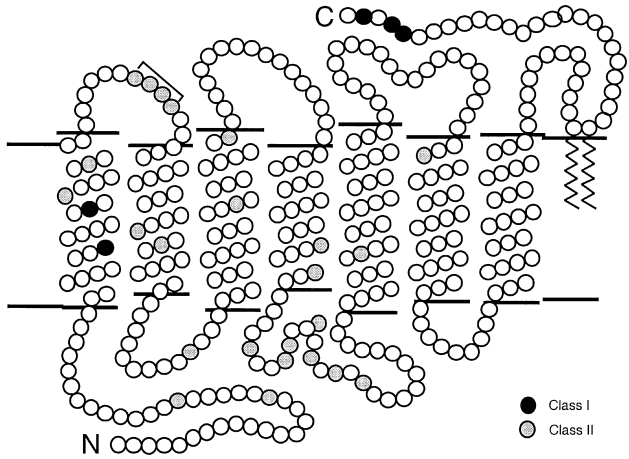
So far, mutations in the rhodopsin gene are the most common cause of RP for which a genetic factor is known. Almost 90 mutations in the rhodopsin gene have been identified in patients with autosomal dominant RP (ADRP; Dryja *et al.,* 1990a,b, 1991; Gal *et al.,* 1991, 1997; Sheffield *et al.,* 1991; Sung *et al.,* 1991a; Inglehearn *et al.,* 1992; Macke *et al.,* 1993). Estimates of the frequency of rhodopsin mutations in ADRP patients range from 10% (Berson, 1996) to as high as 50% (Inglehearn *et al.,* 1998). Most of the rhodopsin mutations found in RP are missense mutations, but a few result in truncations. One null mutation in the rhodopsin gene (nonsense mutation at codon 249) is associated with autosomal recessive RP (ARRP; Rosenfeld *et al.,* 1992). Several mutations in the rhodopsin gene are also found in some cases of congenital stationary night blindness (Dryja *et al.,* 1993).

A. Defective Biosynthetic Processing of Class II Rhodopsin Mutations Associated with Retinitis Pigmentosa

The functional defects of ADRP mutant rhodopsins were first examined in heterologous expression systems by comparison with wild-type rhodopsin (Sung *et al.,* 1991b; 1993; Kaushal and Khorana, 1994). In transfected human embryonic kidney 293S cells and monkey kidney COS-7 fibroblasts, wild-type rhodopsin is produced at high levels and normally glycosylated. Immunocytochemistry shows that rhodopsin is predominantly expressed on the plasma membrane and can be regenerated with 11-*cis*-retinal to produce a functional photopigment with an absorbance spectrum indistinguishable from rhodopsin isolated from retinas, indicating that the protein is correctly folded. Analysis of several dozen different mutant rhodopsins in the same cell culture systems has demonstrated that at least two distinct classes of anomalies exist. The majority (~80%) of the ADRP rhodopsin mutants examined, termed class II, display low levels of protein expression, fail to

produce a functional photopigment on reconstitution with 11-*cis*-retinal, and fail to exit efficiently from the ER (Fig. 3). Further analysis of the class II mutant P23H reveals an abnormal glycosylation pattern on SDS–PAGE and failure to acquire endoglycosidase H resistance, confirming its inability to proceed from the ER to the Golgi apparatus (Sung *et al.,* 1991b). Class II rhodopsin mutations are therefore thought to interfere with correct protein folding. As a result, the mutant rhodopsins are retained in the ER and subsequently degraded. The defects of the class II rhodopsin mutants are reminiscent of pathogenic mutations in other genes such as those in CFTR (Cheng *et al.,* 1990) and the LDL receptor (Goldstein *et al.,* 1985).

The hypothesis that class II rhodopsin mutants are misfolded is supported by *in vivo* evidence in *Drosophila* (Colley *et al.,* 1995). In the *Drosophila* photoreceptor, the rhabdomere is the functional equivalent of the vertebrate OS. It is composed of numerous tightly packed microvilli containing phototransduction machinery. Similar to vertebrates, fly rhodopsin is syn-



Class I	Class II
Photopigment regenerated with 11-*cis*-retinal	Little or no photopigment
Plasma membrane localization	ER localization

FIG. 3 Transmembrane model of human rhodopsin and locations of class I and II ADRP mutations. The horizontal lines represent the lipid bilayer. N and C mark the amino and carboxyl termini of rhodopsin which face the extracellular domain and cytosolic domain, respectively. The zigzags show the palmitoylation signals. Class I and class II mutations exmined are marked (class I, black; class II, gray). The phenotypes of these mutations analyzed in tissue culture cells are summarized.

thesized in the ER, processed in the Golgi, and then delivered to the rhabdomere. The major fly rhodopsin (Rh1) shares 22% amino acid identity with human rhodopsin (O'Tousa *et al.,* 1985; Zuker *et al.,* 1985). Rh1 mutants isolated in a genetic screen for photoreceptor degeneration abnormally accumulate in the ER and are not efficiently transported to the rhabdomere; interestingly, some of these mutations correspond those found in human ADRP (Colley *et al.,* 1995). Biochemically, all heterozygous flies carrying one wild-type and one mutant allele of Rh1 have significantly decreased levels of wild-type rhodopsin compared to animals carrying one wild-type and one null allele of Rh1. This result suggests that the mutant protein may interfere with the biogenesis of wild-type protein in the ER. It is conceivable that rhodopsin maturation involves the formation of a multimeric protein complex and class II rhodopsin mutants have a dominant-negative effect on the formation of this complex. Alternatively, class II rhodopsin mutants may have a dominant-negative effect on the exit of wild-type rhodopsin from the ER by sequestering chaperones or other factors necessary for folding and/or export. In either model, both mutant and wild-type rhodopsins accumulate in the ER and are eventually degraded. A study of several patients with the class II mutation P23H found reduced levels of rhodopsin as measured by imaging fundus reflectometry (Kemp *et al.,* 1992). This is consistent with the hypothesis that the mutant rhodopsin accumulates in the ER and is degraded, but could also be due simply to photoreceptor loss.

The class II human rhodopsin mutant P23H has been transgenically expressed in mouse photoreceptors (Olsson *et al.,* 1992; Roof *et al.,* 1994). Photoreceptor degeneration begins in these transgenic mice as early as postnatal day 10 (P10). Interestingly, no apparent ER accumulation of P23H rhodopsin is observed in these mice. Although much of the P23H mutant rhodopsin, labeled by a human rhodopsin-specific antibody, localizes to the OS by immunofluorescence, some is also found to have accumulated in the IS as well as in the synaptic terminals. A slower accumulation of the P23H mutant protein in the photoreceptor cell body plasma membrane also occurs, becoming detectable by P15. Moreover, other normally OS-localized proteins—transducin and phosphodiesterase—are also mislocalized to the synaptic terminals, suggesting that the transport of these proteins may be coupled to that of rhodopsin (Roof *et al.,* 1994). The accumulation of P23H at the synaptic terminals of photoreceptors was interpreted by the authors as being due to a specific misrouting pathway rather than to a nonspecific accumulation caused by the absence of an OS in partially degenerated photoreceptors. In the latter scenario, newly synthesized rhodopsin might be inserted directly into the IS plasma membrane and retained there or distributed to the synaptic terminal.

The subcellular distribution of class II mutant T17M rhodopsin in a human retina has been examined by immunoelectron microscopy in one patient (Li *et al.,* 1994). Quantitation of immunogold labeling suggests that the OS density of rhodopsin in this RP patient is similar to that in a normal human. However, in some T17M photoreceptors with shortened OSs, rhodopsin labeling is also observed on the ISs, the somata, and the synapses; whether this is secondary to photoreceptor dysfunction, however, is unclear. As in the case of the P23H transgenic mouse, no obviously abnormal intracellular accumulation of rhodopsin or photoreceptor ER membrances can be seen.

B. Defective Outer Segment Trafficking of Class I Rhodopsin Mutations Associated with Retinitis Pigmentosa

In contrast to class II mutations, several ADRP mutants behave like wild-type rhodopsin when expressed in tissue culture cells. These proteins, termed class I mutants, are produced at normal levels, reach the cell surface, and produce a functional photopigment on 11-*cis*-retinal reconstitution. Thus the primary defect in class I mutants does not appear to be incorrect folding and/or stability, as is the case for class II mutants. Two examples of class I mutants—Q344ter (Sung *et al.,* 1994) and P347S (Weiss *et al.,* 1995) show normal light-dependent activation of transducin and normal phosphorylation of the C-terminal domain by rhodopsin kinase. The C-terminal domain of the P347S mutant also binds to arrestin normally after being photoactivated (Weiss *et al.,* 1995). Electrophysiologic recordings of Q344ter transgenic rods (see below) show that their light responses are very similar to those of nontransgenic control rods (Sung *et al.,* 1994). These data are strong evidence that these mutations do not significantly alter the overall conformation of rhodopsin's C-terminus.

Class I mutations are found in a strikingly clustered distribution along the rhodopsin polypeptide (Fig. 3). With the exception of two mutations (G51V and F45L), which are located in the first transmembrane domain, the other known class I mutations are clustered among the last eight amino acids at rhodopsin's carboxyl-terminal cytoplasmic tail. These amino acids are highly conserved among vertebrates (Fig. 4B, Macke *et al.,* 1993). To date, 14 ADRP mutations have been identified in the carboxyl-terminal, cytoplasmic tail of rhodopsin (Fig. 4A, Sung and Chuang, 1999). Although not all of these mutations have been classified in cell culture expression systems, it is tempting to hypothesize that a common defect might be shared among all of these C-terminal ADRP rhodopsin mutations.

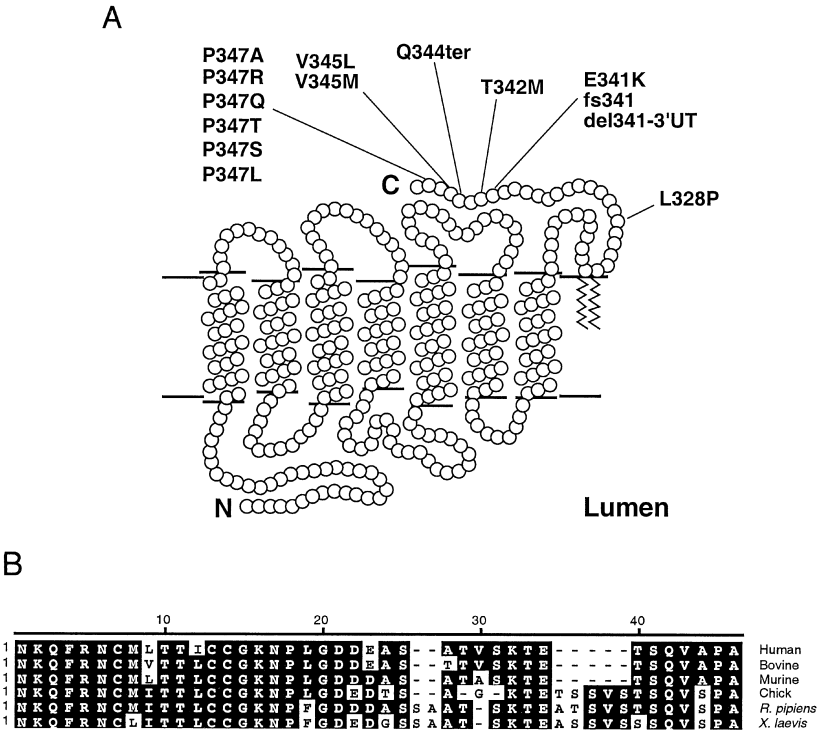


FIG. 4 (A) Fourteen ADRP mutations clustered in the carboxyl-terminal cytoplasmic tail of rhodopsin are shown: L328P, E341K, del341-3′UT (a 42-base pair deletion that removes the last eight codons), fs341 (a frameshift mutation deleting the eight residues beginning with codon 341), T342M, Q344ter (a nonsense mutation removing the last five residues), V345M, V345L, P347A, P347R, P347Q, P347T, P347S, and P347L (Sung and Chuang, 1999). (B) Alignment of the cytoplasmic tail sequences of rhodopsin from several vertebrates (39 residues for mammalian rhodopsins). Two short additional insertions are found in many nonmammalian vertebrate rhodopsins.

Several carboxyl-terminal ADRP mutations have been expressed transgenically. We and our colleagues have examined Q344ter mouse rhodopsin expressed on a wild-type rhodopsin background (Sung *et al.,* 1994). Q344ter transgenic photoreceptors undergo progressive degeneration. The intracellular distributions of the endogenous versus Q344ter transgenic rhodopsin can be distinguished by immunofluorescence using two antibodies: a mouse monoclonal antibody that recognizes the wild-type protein and a rabbit polyclonal antibody that recognizes the truncated mutant protein. These antibodies are directly conjugated to fluorophores, resulting in quantitatively linear labeling intensities. In transgenic retinas with only mild degenerative changes (P15–20), endogenous wild-type rhodopsin is virtually all

localized normally to the OS, but Q344ter mutant rhodopsin is found at significant levels on the plasma membrane enveloping the IS, cell body, and synaptic terminal. The mistargeting is specific to the truncated mutant protein, suggesting that this defect is not caused by transgene overexpression, OS loss, or general disruption resulting from cell deterioration. Furthermore, the unusual distribution of Q344ter rhodopsin occurs prior to the loss of the OS, indicating that this abnormality is likely to be the cause, rather than a result, of photoreceptor death. This experiment further suggests that the C-terminus of rhodopsin contains information directing its polarized OS localization, either by direct targeting to or by retention at the OS.

Two other class I rhodopsin mutants—P347S and P347L—have also subsequently been expressed as transgenes, and both exhibit defects in OS localization. Transgenic mouse lines expressing P347S human rhodopsin suffer from retinal degeneration (Li *et al.,* 1996). However, the visual function of these mice, as measured by an electroretinogram (ERG), is approximately normal prior to the onset of detectable retinal degeneration. This is consistent with the *in vitro* biochemical evidence that P347S appears to be normal in its phototransduction functions (Weiss *et al.,* 1995). At the light microscope level, immunofluorescent labeling with a human rhodopsin-specific antibody (mAb rho 3A6) indicates that the transgenic P347S rhodopsin appears to be predominantly expressed on the OS in a manner indistinguishable from wild-type rhodopsin. However, transmission and scanning electron microscopy reveals extensive accumulation of extracellular vesicles surrounding the IS and OS as well as irregularities in IS morphology. These vesicles are in the submicrometer size in range and are encircled by a single membrane. Higher levels of transgene expression are accompanied by higher levels of extracellular membrane vesicle accumulation. Immunoelectron microscopy shows that these vesicles contain endogenous rhodopsin; it is not clear whether these vesicles also contain mutant rhodopsin. These extracellular membrane profiles are therefore photoreceptor-derived, and presumably originate from aberrant budding from the IS, though no membrane profiles in the process of exocytosis were reported in P347S photoreceptors. Alternatively, assuming that rhodopsin does in fact normally reach the OS via an extracellular route from the IS (see Section IV,D), these vesicles could have accumulated because of failure to correctly fuse with the basal OS.

A pig model of human RP expressing P347L rhodopsin has also been developed. (Petters *et al.,* 1997). In these transgenic pigs, rod cell death occurs around 2 weeks after birth. As with Q344ter rhodopsin in transgenic mice, P347L rhodopsin is mislocalized to the entire rod plasma membrane surrounding the somata and synaptic terminals (Li *et al.,* 1998). Intriguingly, these transgenic rods lack the normally prominent labeling of rhodopsin at

the Golgi complex. Instead, clusters of intracellular membranes containing rhodopsin immunoreactivity, interpreted as autophagosomes, are observed within the IS. Neighboring Müller cells also contain rhodopsin immunoreactivity, suggesting active phagocytosis of debris from degenerating photoreceptors. Rod synaptic terminals also show rhodopsin expression in abnormal filopodia-like processes that extend into the outer plexiform layer. These processes contain no apparent synaptic vesicles or ribbons and make no apparent contact with postsynaptic membranes.

In summary, it is safe to conclude that the three class I ADRP rhodopsin mutants studied as transgenes to date are defective in one or more steps along the vectorial transport pathway to the OS. This finding has two important implications. First, it suggests that the carboxyl-terminal cytoplasmic tail of rhodopsin binds to sorting/targeting machinery involved in rhodopsin trafficking *in vivo.* Second, proper targeting to and retention in the OS of rhodopsin may be important for maintaining photoreceptor function and survival.

VI. Molecular Mechanisms of Rhodopsin Trafficking

The autoradiographic and immunocytochemical studies described in Sections III and IV have provided important information about the transport of rhodopsin in photoreceptors at the subcellular level. The characterization of naturally occurring rhodopsin mutations and their corresponding transgenic animal models, discussed in Section V, has pointed to the presence of sorting/targeting signals on rhodopsin. However, the particular steps in rhodopsin trafficking that are regulated by these singnals and the specific identities of the molecules that mediate these processes have only recently begun to be elucidated. In this section, we will revisit the pathway of rhodopsin trafficking to the OS at the molecular level.

Our understanding of the signals and molecular processes that regulate rhodopsin trafficking in photoreceptors has lagged significantly behind our understanding of protein trafficking in other cell types such as polarized epithelia and hippocampal neurons. At first glance, the highly polarized and compartmentalized photoreceptor would seem to be an attractive model for the study of vectorial protein and lipid trafficking. However, a major impediment to such studies has been the comparative lack of appropriate experimental models of photoreceptor transport. In general, live photoreceptors in intact retinas are not easily accessible for experimental manipulation and live imaging. Biochemical studies of photoreceptors, with the exception of their OSs, are difficult because of contamination from other retinal cell types. Although dissociated mature photoreceptors can be main-

tained in culture for a reasonably long time, their IS and OS plasma membranes quickly fuse with one another, resulting in the diffusion of rhodopsin throughout the entire photoreceptor and a consequent loss of polarity (Townes-Anderson *et al.,* 1985; Spencer *et al.,* 1988; Townes-Anderson, 1995). Although the mechanism of this fusion is uncertain, it is probable that the loss of membrance asymmetry in dissociated photoreceptors is due in part to the lack of adjacent supporting RPE and/or Müller cells. Additional difficulties in using cultured photoreceptors include the small size of mammalian photoreceptors and their extremely high metabolic demands. Dissociated chick embryonic retinal cells can be cultured (Saga *et al.,* 1996); however, only a fraction of these cells develop into rhodopsin-expressing cells and even fewer express rhodopsin in a polarized manner in rudimentary OS-like structures. Whether such cells can be used as models of protein transport in mature photoreceptors is doubtful.

Although transgenic and knockout technologies have proved valuable for studying photoreceptor protein transport *in vivo,* they also possess significant limitations, not the least of which is the considerable investment in time and labor required. Moreover, these techniques are not well suited to studying the kinetics of vectorial photoreceptor protein trafficking. Alternative experimental models of photoreceptor transport will prove to be useful complements to transgenic/knockout techniques. In this section we will discuss in passing several *in vitro* and *in vivo* systems of rhodopsin trafficking, established in recent years, that have provided new approaches to studying the molecular mechanisms of rhodopsin transport.

A. Identification of Rhodopsin Targeting Signals

Proteins sorted or targeted to specific intracellular compartments generally contain addressing information within their own peptide sequences. For a number of receptors, subcellular targeting is mediated by sequences contained within their cytosolic tails. Examples include endocytosis of LDL (Davis *et al.,* 1987) and transferrin receptors (Rothenberger *et al.,* 1987) and the internalization and lysosomal trafficking of the mannose-6-phosphate receptor (Lobel *et al.,* 1989). All basolateral sorting signals described to date also reside on the cytoplasmic domains of proteins (Mostov *et al.,* 1986; Roth *et al.,* 1987; Hunziker *et al.,* 1991). The observed mislocalization of class I carboxyl-terminal rhodopsin mutants in transgenic photoreceptors, as described in Section V,B, provided the first indication that the C-terminus of rhodopsin may encode signals that direct its polarized trafficking. However, which step(s) in rhodopsin trafficking this carboxyl-terminus regulates cannot be readily determined from these studies.

1. Structural Analysis of Rhodopsin's Carboxyl Terminus

We have found no significant homology between the C-terminus of rhodopsin and any previously described domain or sorting signal. However, sorting signals and protein–protein interaction domains are frequently either short and/or fairly loosely conserved. Moreover, the sorting signal within rhodopsin's C-terminus may be conformationally dependent. These possibilities are likely to limit the usefulness of current algorithms for analyzing rhodopsin's C-terminus for novel interaction domains.

The hypothesis that the C-terminus of rhodopsin contains ordered structural domains is supported by ^{1}H nuclear magnetic resonance (NMR) and circular dichroism spectroscopic analyses of synthetic rhodopsin C-terminal peptides [C-terminal 33 residues (Yeagle *et al.,* 1995) or 43 residues (Yeagle *et al.,* 1996)]. These studies have suggested that the cytoplasmic tail of rhodopsin forms two distinct structural domains; the first extends from the end of the seventh transmembrance helix to the palmitoylation sites at residues 322 and 323, creating a structurally ordered fourth cytoplasmic loop, followed by a partial β sheet that extends to the C-terminus. Palmitoylation is not required for the formation of these two structural domains. This may explain why removal of these palmitoylation sites does not inhibit the activation of transducin by rhodopsin (Karnik *et al.,* 1993) and also does not affect its apical targeting in MDCK cells (see Section VI,A,3; Chuang and Sung, 1998).

2. Cell-Free Vesicle Formation Assay

Studies using a cell-free vesicle formation assay developed by Deretic and co-workers suggest that the carboxyl terminus of rhodopsin plays a role in post-Golgi vesicle formation and trafficking (Deretic *et al.,* 1996, 1998). In these studies, frog retinas are isolated and metabolically pulse-labeled in culture for 1 hr. Following OS removal, the rest of the retina is homogenized and centrifuged. The resulting postnuclear supernatant, which contains photoreceptor biosynthetic membrances, is chased for 2 hr in the presence of an ATP-regenerating system, followed by equilibrium density gradient centrifugation. After the chase, a significant proportion of metabolically labeled rhodopsin is found to be distributed in low buoyant density fractions containing vesicles resembling those generated *in vivo* on the basis of their similarity in buoyant density (1.09 g/ml), morphology, and protein composition (see Section IV,B). The density and composition of these light fractions are distinct from those of Golgi fractions and the sialyltransferase-positive TGN fractions. The appearance of rhodopsin in post-Golgi vesicle fractions is significantly inhibited by the addition of a monoclonal antibody (mAb 11D5) which recognizes the carboxyl-terminal 9 residues of rhodop-

sin (Deretic *et al.,* 1996). Moreover, addition of peptides corresponding to the carboxyl-terminal 25 amino acids of bovine or of frog rhodopsin also similarly reduce the appearance of rhodopsin in the post-Golgi fractions (Deretic *et al.,* 1998). In contrast, these peptides have no apparent effect on the exit of newly synthesized rhodopsin from the ER or its progression through the Golgi, indicating that this inhibition is specific to the TGN. Most importantly, mutant peptides corresponding to ADRP mutations (Q344ter, V345M, or P347S) fail to prevent newly synthesized rhodopsin from entering the post-Golgi membrane fractions.

These findings strongly suggest that rhodopsin's C-terminus contains a signal required for sorting rhodopsin into specific post-Golgi membranes at the level of the TGN, and that this signal is dependent on the C-terminal five amino acids—QVAPA in most mammals and QVSPA in frogs, fish, and lampreys. The signal functions perhaps by regulating rhodopsin recruitment into specific vesicle budding sites and/or by regulating the budding event itself. Although the carboxyl termini of most amphibian rhodopsins contain extra amino acids at the seventh and eleventh positions relative to the C-termini of mammalian rhodopsins (Fig. 4B), the inhibitory abilities of both frog and bovine sequences in the frog retinal cell-free system imply that these additional sequences are not required for rhodopsin sorting at the TGN in amphibians.

3. Studies in Polarized Epithelial Cells

Madin-Darby canine kidney (MDCK) epithelial cells are a useful and widely used model for studying vectorial membrance trafficking (for review, see Rodriguez-Boulan and Powell, 1992). MDCK cells in cultures form polarized monolayers with distinct apical and basolateral surfaces. The asymmetric membrance structure of MDCK cells is established and maintained both by vectorial sorting and targeting of molecules on transit through the TGN as well as by tight junctions—structures that prevent the free diffusion of membrance proteins between the apical and basolateral domains. The polarized sorting and/or transport of many membrane proteins in MDCK cells has been found to be tightly regulated via the recognition of specific sorting signals. Our laboratory has shown that wild-type rhodopsin is targeted to the apical plasma membrance on stable expression in polarized MDCK cells (Chuang and Sung, 1998). Pulse–chase labeling experiments show that rhodopsin targeting occurs directly via the TGN. Truncated rhodopsin lacking the 32 C-terminal residues exhibits a nonpolar steady-state distribution, suggesting that the terminal 32 residues contain required apical targeting information. Although this truncated rhodopsin also lacks the palmitoylation sites, a nonpalmitoylated site-directed mutant rhodopsin has an apical distribution indistinguishable from wild-type rhodopsin, indi-

cating that palmitoylation is not essential for rhodopsin's apical sorting in MDCK cells. Finally, addition of the entire carboxyl-terminal cytoplasmic tail (39 residues) of rhodopsin redirects the basolateral membrane protein CD7 to the apical membrane, demonstrating that the cytoplasmic C-terminus of rhodopsin is an independent apical sorting determinant in MDCK cells. Moreover, these results imply that cytosolic sorting components may exist for the apical targeting of rhodopsin, analogous to those described for basolaterally targeted proteins (Nelson, 1992).

A simple model to explain these results is that the apical membrance of MDCK cells is topologically equivalent to the apical IS plasma membrane of photoreceptors. Thus, rhodopsin is sorted ''apically'' in both systems. Consistent with this notion is the observation that Na^+/K^+-ATPase, which accumulates basolaterally in MDCK cells (Hammerton *et al.,* 1991; Gottardi and Caplan, 1993), is exclusively expressed on the lateral plasma membrane of the photoreceptor IS (Schneider and Kraig, 1990). Indeed, it has been proposed that common mechanisms are used for sorting proteins to the apical/basolateral surfaces of polarized epithelia and to the axonal/dendritic regions of neurons (Dotti *et al.,* 1991). However, this model does not fully explain rhodopsin trafficking in MDCK cells versus photoreceptors: whereas a short C-terminal deletion of 5 amino acids is sufficient to alter rhodopsin's OS localization in transgenic photoreceptors (Sung *et al.,* 1994), a longer deletion is necessary to abolish its apical sorting in MDCK cells. It is conceivable, though, that the trafficking pathway to the apical plasma membrane in MDCK cells does not fully replicate the pathway to the OS in photoreceptors. Since photoreceptors are more complicated in their compartmentalization than conventional neurons or polarized epithelia, rhodopsin trafficking in MDCK cells is likely to only involve a subset of the transport steps that occur in photoreceptors. It may be that rhodopsin contains two distinct apical targeting signals required for OS targeting—one at the distal C-terminus and one at the proximal part of its cytoplasmic tail—and that the distal signal is not recognized or not required for apical sorting in MDCK cells. This hypothesis is compatible with structural data suggesting the existence of two distinct domains in rhodopsin's C-terminus (see Section VI,A,1). Although further studies need to be performed, polarized epithelial cells may turn out to be good models for at least a subset of the sorting/targeting steps that rhodopsin encounters in the photoreceptor.

B. Candidate Proteins Involved in Rhodopsin's Polarized Transport

The evidence discussed in the previous Section (VI,A) suggests that one or more sorting/targeting signals reside in the cytoplasmic carboxyl-terminal

tail of rhodopsin. It is therefore extremely likely that there are proteins involved in the trafficking of rhodopsin that interact, directly or indirectly, with these targeting signals. Such proteins could conceivably regulate rhodopsin trafficking at one or more of the steps along its route to the OS, and they will be the subject of this section. It must be noted, though, that there are also likely to be other proteins present on rhodopsin-bearing transport vesicles that regulate aspects of rhodopsin trafficking independently of rhodopsin itself.

1. Sorting/Budding

a. Coat Protein Complexes Two main types of vesicle coat protein complexes—clathrin coated and nonclathrin coated—are involved in vesicle formation at several stages of the secretory and endocytic pathways (reviewed in Schekman and Orci, 1996). In the case of clathrin-coated vesicles, clathrin binds to the donor membrane (either TGN or plasma membrane) via adaptor complexes that couple the selection of cargo molecules to coat assembly (Pearse and Robinson, 1990; Mellman, 1996). The cargo molecules are then segregated at the donor membrane into coated pits and subsequently packaged into clathrin-coated vesicles for delivery. AP-1 is the adaptor used at the TGN, and AP-2 is the adaptor used at the plasma membrane (Le Borgne and Hoflack, 1998). Evidence has shown that there is a direct interaction between tyrosine-and leucine-based sorting signals on the cytoplasmic tails of transmembrane proteins and the adaptor complexes AP-1 and AP-2 (Ohno *et al.,* 1995; Rodionov and Bakke, 1998). This interaction is likely to be the first step in the formation of clathrin-coated vesicles.

In contrast to the clathrin coat complex, the nonclathrin coat complexes known as coatomer (COP) coats are generally thought to be involved in transport through the early secretory pathway such as ER–Golgi and intra-Golgi trafficking rather than in post-Golgi trafficking (Kreis *et al.,* 1995). As with clathrin coat complexes, coatomer is also likely to play an important role in the sorting and concentration of cargo proteins by interacting with their cytoplasmic domains (Fiedler *et al.,* 1996; Kuehn and Schekman, 1997).

Additional coat protein complexes, in addition to clathrin and COP coats, also have been described and are possibly involved in the formation of secretory vesicles that bud from the TGN (de Almeida *et al.,* 1993; Ladinsky *et al.,* 1994; Simpson *et al.,* 1996). However, the actual functions of these putative coat proteins in post-TGN vesicle formation remain unclear.

The question of whether coat proteins participate in the formation of rhodopsin-bearing vesicles at the TGN has not been fully explored. Although post-Golgi transport vesicles carrying rhodopsin do not contain clathrin and lack discernible coats (Papermaster *et al.,* 1979; Deretic and

Papermaster, 1991), a transient role for coat proteins in cargo selection and vesicle budding at the rod TGN cannot be ruled out at the present.

b. Small GTP-Binding Proteins—Rabs Rab proteins comprise a large family of over 40 small, monomeric GTP-binding proteins that localize to distinct membrane compartments by reversibly associating with donor membranes and transport vesicles. They appear to regulate vesicle formation as well as docking and fusion with target membrances; however, much remains unknown about the precise mechanisms by which these activities are accomplished (Novick and Zerial, 1997). GTP-bound Rab (Rab-GTP) is membrane bound and catalytically active. Hydrolysis of GTP to GDP occurs on or after fusion with target membranes, and the inactive form of Rab-GDP is released from the membrane. Rab-GDP dissociation inhibitor (Rab-GDI) forms a complex with Rab-GDP in the cytosol and prevents GDP release until it encounters a specific nucleotide exchange factor (Simons and Zerial, 1993, Ferro-Novick and Novick, 1993).

Multiple Rab proteins (Rab3, Rab6, Rab8, and Rab11) are associated with rhodopsin-bearing post-Golgi vesicles isolated from frog retinas or produced by the cell-free budding assay described in Section IV,B (Deretic and Papermaster, 1993; Deretic *et al.,* 1995, 1996). Moreover, immunoelectron microscopy reveals the presence of Rab6 on transport vesicles clustered near the base of the CC; interestingly, Rab6 immunoreactivity is also seen at the base of the OS (Deretic and Papermaster, 1993). In the cell-free budding assay, the addition of recombinant Rab-GDI to the reaction mixture following preincubation with excess GDP abolishes the formation of rhodopsin-bearing post-Golgi membrance vesicles, accompanied by the arrest of ''immature'' glycosylation forms of rhodopsin in the fractions containing ER and Golgi membranes (Deretic *et al.,* 1996). However, it is not clear whether the inhibitory effect of Rab-GDI on post-Golgi vesicle formation is due to a specific block of TGN-budding or a secondary effect arising from inhibition of ER–Golgi and intra-Golgi transport, since Rab-GDI has little specificity and can interact with a broad range of Rab proteins. It would therefore be useful to examine whether dominant-negative mutants of specific Rab proteins can also block newly synthesized rhodopsin in the Golgi.

2. Directional Translocation

The importance of microtubule-based motility for the transport of rhodopsin through the IS has been challenged by Vaughan *et al.* (1989). In this report, the authors assay the rate of disk morphogenesis in explanted frog eyecups incubated in culture medium containing Lucifer Yellow, which results in the incorporation of dye into the lumina of newly formed and

sealed rod photoreceptor disks. At different time points, the retinas are washed free of unincorporated dye before being fixed and sectioned. Finally, the heights of the fluorescently labeled bands in the rod OSs, representing disks sealed during the labeling period, are measured. The mean height of the labeled bands (~1 μm) after a fixed period of labeling (~10–11 hr) is used as a surrogate measure for the rate of rhodopsin transport through the IS during this period. The addition of various microtubule-depolymerizing drugs such as nocodazole and colchicine resulted in band height decreases relative to controls, although the decreases were not statistically significant. This was interpreted to mean that rhodopsin transport to the OS is not dependent on IS microtubule integrity. However, the limited resolving ability of light microscopy for such short lengths, as well as the short time course of treatment, mean that their experimental system may have simply lacked sufficient sensitivity to detect a partial inhibition of rhodopsin transport at statistically significant levels.

On the other hand, significant insight has come from studies in many cell systems showing that microtubules serve as highways for the travel of transport intermediates between the ER and Golgi, and probably further along the secretory pathway as well (reviewed by Bloom and Goldstein, 1998). Observations *in vitro* and *in vivo* suggest that Golgi and Golgi-derived membranes can bind to microtubules, possibility via motor proteins. Work on organelle motility has led to the general principle that kinesin is the motor for movement toward microtubule "plus" ends, whereas cytoplasmic dynein is the motor for movement toward "minus" ends (reviewed in Hirokawa, 1998). Microtubules are known to be required for the axonal transport of molecules in neurons (reviewed in Sheetz *et al.,* 1989). They are also required for the efficient transport of post-TGN vesicles and for the transcytosis of vesicles from the basolateral membrane to the apical cytoplasm in polarized epithelia (Parczyk *et al.,* 1989; Gilbert *et al.,* 1991). We and our co-workers have found that microtubule-disrupting drugs alter the normally apical distribution of rhodopsin in MDCK cells, causing rhodopsin to became uniformly distributed (C.-H. Sung and J.-Z. Chuang, unpublished result, 1998). This observation suggests that the apical transport of rhodopsin in MDCK cells also requires intact microtubules.

a. Cytoplasmic Dyneins As discussed in Section II,B,1, cytoplasmic microtubules in fish photoreceptors are oriented with uniform polarity, with their "minus" ends facing the OS and "plus" ends facing the synaptic terminal (Fig. 1B; Troutt and Burnside, 1988). Thus, from the point of view of the Golgi apparatus located in the proximal IS, the cytoplasmic microtubules directed toward the OS are arranged with a polarity opposite to those directed toward the axon terminal. Therefore, cytoplasmic dynein, which can translocate membrane vesicles along microtubules to their "mi-

nus'' ends, is a promising candidate motor for the transport of rhodopsin-laden vesicles across the IS to the base of the CC. Using a yeast two-hybrid screen for proteins that interact with the carboxyl terminus of rhodopsin, we isolated Tctex-1 (*t*-complex testis expressed-1), which had been previously demonstrated to be a 14-kDa cytoplasmic dynein light chain (King *et al.,* 1996a; Harrison *et al.,* 1998). Each multisubunit cytoplasmic dynein complex consists of two heavy chains containing the ATPase and motor activities (~530 kDa; Vallee and Shpetner, 1990), two or three intermediate chains (~74 kDa), a group of light intermediate chains (~52–61 kDa; Gill *et al.,* 1994), and several light chains (8, 14, and 22 kDa), including Tctex-1 (King *et al.,* 1996a,b).

In fibroblasts, Tctex-1 is concentrated at the Golgi apparatus as well as along microtubules, indicating a potential function for Tctex-1 in Golgi or post-Golgi trafficking along microtubules (Tai *et al.,* 1998). Our recent work has demonstrated that rhodopsin's C-terminal cytoplasmic tail interacts directly and specifically with Tctex-1 in a number of *in vitro* binding assays (Tai *et al.,* 1999). Several naturally occurring carboxyl-terminal rhodopsin mutants (V345M, P347S, P347L, and Q344ter) have markedly lower affinity for Tctex-1, indicating that the rhodopsin–Tctex-1 interaction is likely to be physiologically relevant. Rhodopsin-containing membrane vesicles, prepared by sonicating purified OSs, associate with microtubules via cytoplasmic dynein in a Tctex-1-dependent manner. This can be demonstrated by microtubule cosedimentation and motility assays. Immunoelectron microscopic examination of mouse photoreceptors indicates that Tctex-1 is present on the Golgi apparatus, transport vesicles, and at the apical membrane of the IS, and that Tctex-1 colocalizes with rhodopsin on IS transport vesicles. Interestingly, Tctex-1 labeling is absent from the CC as well as the OS, suggesting a mechanism for Tctex-1/cytoplasmic dynein release from rhodopsin. Collectively, these results strongly argue that cytoplasmic dynein is involved in the vectorial transport of rhodopsin in the photoreceptor from the proximal Golgi region to the apical IS and that rhodopsin functions as a membrane receptor for the cytoplasmic dynein complex. Furthermore, our results indicate that the reduced ability of rhodopsin mutants to associate *in vitro* with Tctex-1, and therefore with cytoplasmic dynein, may lower the efficiency of their vectorial targeting *in vivo.* This may explain the trafficking defects of C-terminal rhodopsin mutants observed in transgenic models of RP (see Section V,B).

b. Kinesins The transport of rhodopsin between the IS and OS is another step that may involve motor proteins, assuming that rhodopsin does indeed travel in the plasma membrane of the CC and not by an extracellular route. Members of the kinesin and myosin families have been localized to the CC.

Kinesin superfamily proteins (KIFs) comprise a large number of microtubule-based motors sharing a conserved motor domain. Most KIFs move toward the ''plus,'' or fast-growing, ends of microtubules (reviewed in Hirokawa, 1998). Because of this directionality, kinesins are good candidates for translocating rhodopsin from the base of the CC (the ''minus'' ends of the axonemal microtubules) to the OS (the ''plus'' ends of the axonemal microtubules) (see Section II,B,1). Photoreceptors probably express multiple kinesins: using degenerate primers directed against conserved kinesin motor domain sequences, 11 different kinesins can be identified by RT–PCR in fish neural retina (Bost-Usinger *et al.,* 1997). It was not determined, though, how many of these kinesins are expressed specifically in photoreceptors. Antibodies recognizing conserved kinesin epitopes strongly label the CC and axonemal microtubules in sunfish photoreceptors and purified bovine photoreceptor axonemes by immunofluorescence and immunoelectron microscopy (Beech *et al.,* 1996; Muresan *et al.,* 1997). Furthermore, an affinity-purified antibody against KIF3A, a neuronal kinesin, immunolabels the basal bodies, CC/axonemal microtubules, and the ISs of fish photoreceptors (Beech *et al.,* 1996). The subcellular localization of certain kinesins to the CC in photoreceptors is intriguing and indicates their potential role in the translocation of materials between the rod IS and OS; this has been proposed to occur in a manner similar to the kinesin-driven intraflagellar transport of new components to the growing tip of motile cilia (Rosenbaum *et al.,* 1999). However, there is no direct evidence to date supporting a role for kinesins in IS–OS transport.

C. Myosin VIIa Another candidate motor for the transport of rhodopsin from the IS to the OS via the CC is myosin VIIa. Myosin VIIa is a member of the growing family of unconventional myosins (for review, see Hasson and Mooseker, 1996; Mermall *et al.,* 1998). Although myosin VIIa motor activity has yet to be demonstrated biochemically, the presence of a conserved myosin motor domain at its N-terminus indicates that myosin VIIa is likely to be a functional actin-based motor. The possibility that myosin VIIa plays a role in photoreceptor trafficking has been brought to attention by the identification of mutations in myosin VIIa in patients with Usher syndrome type 1B (Weil *et al.,* 1995). Usher syndrome is an autosomal recessive disease characterized by the combination of RP and profound hearing loss as well as vestibular dysfunction. While an initial report (Hasson *et al.,* 1995) did not find any myosin VIIa labeling in rat photoreceptors, a later report (Liu *et al.,* 1997b) demonstrates, using two different myosin VIIa antibodies, that myosin VIIa is concentrated in the CC of human and rodent photoreceptors, but not in other regions of the photoreceptor. Immunoelectron microscopic analysis further reveals that myosin VIIa is localized near the ciliary plasma membrane and is absent from the center

of the ciliary axoneme (Liu *et al.,* 1997b; Wolfrum and Schmitt, 1999). Although the colocalization of myosin VIIa with rhodopsin (see Section IV,D) on the CC plasma membrane is intriguing, it remains unknown whether myosin VIIa is directly involved in rhodopsin transport to the OS. If myosin VIIa is indeed involved in rhodopsin translocation, it would be of great interest to determine whether this is mediated by a direct interaction between rhodopsin and myosin VIIa. Alternative functions for myosin VIIa at the CC can be envisioned, such as maintenance of a diffusion barrier between the IS and OS.

3. Plasma Membrane Fusion

Virtually nothing is known about how rhodopsin-bearing post-Golgi vesicles dock and fuse with the IS apical plasma membrane. It is probable that the specificity of rhodopsin-bearing vesicle fusion with the plasma membrane is regulated in part by Rab proteins. Rab proteins have been thought to regulate membrane fusion in part by regulating the interaction of v-SNAREs with their cognate t-SNAREs. Several Rabs are associated with rhodopsin-bearing post-Golgi vesicles (Section VI,B,1,b; Deretic and Papermaster, 1993; Deretic *et al.,* 1995). The specific roles of these Rabs in membrane docking/fusion of rhodopsin-bearing vesicles remain to be determined.

It is probable that rhodopsin-containing membrane vesicles carry one or more v-SNAREs that help determine their fusion specificity. It is generally hypothesized that the specificity of vesicle docking and fusion with target membranes is in large part determined by their respective SNARE molecules (reviewed in Söllner, 1995; Pfeffer, 1996). That is, carrier vesicles bearing specific v-SNAREs are thought to be competent to dock and fuse only with target membranes bearing the appropriate cognate t-SNAREs. It is not known which v-SNAREs, if any, are carried by rhodopsin-bearing vesicles. It would also be interesting to determine which t-SNAREs are expressed at the apical plasma membrane/periciliary ridge complex of the IS and whether they differ from the t-SNAREs expressed on the lateral IS and cell body plasma membranes. The expression of the appropriate t-SNAREs for rhodopsin-bearing vesicle fusion on the apical, but not the lateral, IS plasma membrane would provide an additional mechanism for directing the vectorial flow of newly synthesized rhodopsin molecules toward the OS.

C. Implications for the Transport of Other Outer Segment Components

Although rhodopsin is by far the major protein constituent of the rod OS, the other protein constituents of the OS, as well as the lipids that make

up the OS plasma and disk membranes, must also be transported to the OS after their synthesis in the IS. It is interesting to note that the targeted disruption of both rhodopsin alleles in mice completely blocks rod OS formation (Humphries *et al.,* 1997; Lem *et al.,* 1999). Although this phenomenon could conceivably reflect a specific requirement for rhodopsin in OS morphogenesis, an attractive alternative hypothesis is that the vectorial trafficking of at least certain proteins and/or lipids required for OS formation to the OS is achieved by cotransport with rhodopsin. In this model, post-Golgi vesicles carrying components intended for the OS would not be efficiently directed to the OS—or might not even be correctly sorted at the TGN—in the absence of rhodopsin's sorting/targeting signals. In support of this hypothesis is the finding that while OS formation is not significantly impaired in photoreceptors expressing a single wild-type rhodopsin allele (Humphries *et al.,* 1997; Lem *et al.,* 1999), photoreceptors expressing transgenic P347S class I mutant rhodopsin on a rhodopsin null background fail to develop OSs (T. Li, personal communication, 1999).

1. Transport of Other Newly Synthesized Outer Segment Proteins

a. Outer Segment Integral Membrane Proteins The most efficient mechanism for the delivery of other integral membrane proteins to the OS would presumably be by cotransport with rhodopsin. However, it has been reported that the OS integral membrane protein peripherin/*rds* may in fact follow a different transport pathway from that of rhodopsin (Fariss *et al.,* 1997). In a retinal detachment model of photoreceptor degeneration, rhodopsin and peripherin/*rds* are found to have very distinct subcellular distributions: rhodopsin accumulates in the cell body plasma membrane, whereas peripherin/*rds* accumulates in cytoplasmic vesicles in the IS (Fariss *et al.,* 1997). Surprisingly, immunoelectron microscopy indicates that these peripherin/*rds*-containing IS vesicles appear to be associated with the ciliary rootlet (see Section II,B,1), an association that has not been reported for rhodopsin-containing vesicles. These findings indicate that the two proteins may follow separate transport pathways to the OS after synthesis. However, these results were obtained primarily in degenerating photoreceptors; it will be important to determine whether rhodopsin and peripherin/*rds* indeed localize to distinct IS vesicles in healthy photoreceptors. It will also be of great interest to determine if there are any other OS membrane-associated proteins that are not cotransported with rhodopsin to the OS and, if so, whether they travel with peripherin/*rds.*

It is curious that while rhodopsin is distributed on both OS disk and plasma membranes (Jan and Revel, 1974; Papermaster *et al.,* 1978; Besharse and Pfenninger, 1980; Nir and Papermaster, 1983), most other OS integral

membrane proteins are expressed preferentially on either the plasma membrane or the disk membrane. For example, the cGMP-gated cation channel (Cook *et al.,* 1989) and the Na^+–Ca^{2+} exchanger (Reid *et al.,* 1990) are largely restricted to the OS plasma membrane. Other OS integral membrane proteins such as peripherin/*rds* (Molday *et al.,* 1986), rom-1 (Bascom *et al.,* 1992), and the ABCR/RIM protein (Illing *et al.,* 1997) preferentially accumulate at the rim of the disk membrane. How these proteins are sorted into distinct membrane subdomains of the OS is unclear.

b. Outer Segment Peripheral Membrane Proteins Subunits of the peripheral membrane proteins transducin and cGMP phosphodiesterase are present on rhodopsin-bearing post-Golgi vesicles, suggesting that they are cotransported with rhodopsin (Deretic and Papermaster, 1991); furthermore, α-transducin and cGMP phosphodiesterase are mislocalized along with rhodopsin in transgenic mouse photoreceptors expressing P23H rhodopsin (Roof *et al.,* 1994; see Section V,A). However, since transducin and phosphodiesterase subunits are not integral membrane proteins, they are presumably synthesized by free ribosomes in the IS, rather than by ER-associated polysomes. Subsequent acylation, isoprenylation, and intersubunit interactions allow these proteins to associate with membranes (Kokame *et al.,* 1992; Neubert *et al.,* 1992; Qin and Baehr, 1994). It is not known how these proteins then find their way onto rhodopsin-bearing post-Golgi vesicles. In addition, it is not known whether these proteins are completely dependent on cotransport with rhodopsin in order to reach the OS, or whether these proteins also contain their own OS trageting signals. The latter possibility is suggested by the apparent ability of transducin to move between the OS and IS independently of rhodopsin (see next section).

2. Light-Dependent Movement of Proteins between Inner and Outer Segments

On its incorporation into OS disks, rhodopsin is retained in the OS until it is shed and phagocytosed by the RPE. In contrast, it appears that several other proteins that were once also assumed to be resident OS proteins in fact shuttle between the IS and OS in a light-dependent manner (Broekhuyse *et al.,* 1985; Philp *et al.,* 1987; Mangini and Pepperberg, 1988; Whelan and McGinnis, 1988). For example, in dark-adapted rodent photoreceptors, arrestin, which is a soluble protein involved in the termination of rhodopsin signaling, is located primarily in the distal IS. On light exposure, arrestin rapidly moves from the IS to the OS. On the other hand, the α and $\beta\gamma$ subunits of transducin, primarily located in the OSs of dark-adapted rods, move in the opposite direction on light stimulation. Movement of these proteins has been demonstrated by immunocytochemistry of retina sections and by immunoblotting of purified rod OSs from light-adapted versus dark-

adapted retinas. The redistribution of these proteins between the IS and OS occurs very rapidly on light exposure. For example, a detectable shift in arrestin localization to the base of the OS is already evident after 30 sec of exposure to light (Whelan and McGinnis, 1988). Arrestin returns to the IS much more slowly after a return to darkness.

The speed and bidirectionality of this striking phenomenon suggest the presence of an active transport mechanism between the IS and OS. It is conceivable, though at present only a matter of speculation, that these proteins are shuttled between these two compartments of the photoreceptor by microtubule- or actin-based motor proteins (Section VI,B,2).

3. Transport of Newly Synthesized Lipids to the Outer Segment

Newly synthesized lipids appear to be able to follow more than one pathway to the OS. Rod OS disk membranes have a lipid composition quite different from typical cell membranes: they are rich in polyunsaturated fatty acids (about 50 mol%), particularly docosahexaenoic acid (DHA, 22:6ω3; Fliesler and Anderson, 1983; Choe and Anderson, 1990), and low in cholesterol (about 6 mol%; Fliesler and Schroepfer, 1982). Metabolic labeling of retinas using labeled fatty acids has shown that rod OS membranes are renewed by two mechanisms (Bibb and Young, 1974; Young, 1976). The first is the incorporation of newly synthesized phospholipids into new disk membranes at the base of the OS (membrane replacement). The second involves remodeling and turnover of phospholipids in mature disk membranes (molecular replacement). Pulse–chase experiments in frog retinas using [^{3}H]DHA, followed by subcellular fractionation, have suggested that some [^{3}H]DHA-phospholipds reach the OS mainly in association with rhodopsin-bearing post-Golgi vesicles (membrane replacement) whereas others appear to become rapidly incorporated into the OS by molecular replacement (Rodriguez de Turco *et al.,* 1997). Finally, various pharmacological agents that block rhodopsin synthesis or transport to the OS do not block the transport of newly synthesized lipids to the OS (Matheke *et al.,* 1984; Fliesler and Basinger, 1987; Wetzel *et al.,* 1993; Fliesler *et al.,* 1995). Thus, the transport of rhodopsin and at least certain OS lipids can be uncoupled, albeit under specialized conditions.

VII. Rhodopsin Trafficking and Photoreceptor Survival

A. How Do Rhodopsin Trafficking Defects Compromise Cell Viability?

One of the most important questions in the study of photoreceptor degeneration is how RP, a single clinical and histopathologic entity, can be so

genetically and biochemically heterogeneous (see Section V). Several groups have shown that apoptotic cell death appears to be a common final end point in many different animal models of photoreceptor degeneration, including transgenic mice carrying P347S and Q344ter mutant rhodopsins (Chang *et al.,* 1993; Lolley *et al.,* 1994; Portera-Cailliau *et al.,* 1994; Tso *et al.,* 1994). However, it is not known how photoreceptors carrying specific mutations decide to enter apoptosis. At least two possibilities can be imagined. The first is that the biochemical signaling pathways that lead a photoreceptor to initiate apoptosis are specific to the particular gene affected—or even to the particular mutation in a given gene. In this scenario, not only the entry points to apoptosis but perhaps also the downstream effector cascades that would be activated (e.g., Bcl-2 independent versus Bcl-2 dependent) might vary from gene to gene or mutation to mutation. Alternatively, apoptosis could simply be a ''nonspecific'' response to diverse forms of stress in photoreceptors that would result in the stereotyped activation of a single apoptotic pathway.

Retinal degeneration caused by dominant rhodopsin mutations is unlikely to result from haploinsufficiency. Humans heterozygous for a putative rhodopsin null mutant lacking the last two transmembrane domains and the 11-*cis*-retinal attachment site do not develop retinal degeneration (Rosenfeld *et al.,* 1992). Similarly, little retinal degeneration is found in mice heterozygous for a rhodopsin null mutation (Humphries *et al.,* 1997; Lem *et al.,* 1999).

Class II ADRP mutations (see Section V,A) are believed to result in rhodopsin misfolding and therefore retention in the ER. How this leads to cell death is not clear; perhaps the presence of misfolded rhodopsin in the ER interferes with the normal biosynthetic function of the ER by an unknown mechanism, or the metabolic cost of degrading ER-accumulated rhodopsin may be unacceptably high. Normal rods are spared the metabolic costs of degrading visual pigment because their rhodopsin is degraded exclusively by the RPE. A second, nonmutually exclusive mechanism of pathology might result from the transport of some of the mutant rhodopsin to the OS, where it could interfere with phototransduction. This conjecture is supported by the observations that many patients with ADRP have a marked decrease in the rate of dark adaptation as well as an elevation in the dark-adapted threshold (Newsome, 1988; Jacobson *et al.,* 1991; Kemp *et al.,* 1992) and that some P23H rhodopsin can be found in the OSs of transgenic mouse photoreceptors (Olsson *et al.,* 1992; Roof *et al.,* 1994). A third potential mechanism is that the abnormal accumulation of rhodopsin in other regions of the rod might switch on some unusual signal transduction pathway that subsequently leads to the execution of apoptosis.

In contrast to the class II mutants, the primary defect of class I rhodopsin mutants appears to lie in their post-Golgi trafficking to the OS (see Section V,B). Q344ter and P347L mutant rhodopsins are mislocalized to the IS

and cell body plasma membranes in transgenic animals even before the onset of degeneration (Sung *et al.,* 1994; Li *et al.,* 1998). Thus, the mislocalization of these rhodopsins is likely to be related to the cause of, rather than the consequence of, cell dysfunction. The finding that P347S transgenic mouse retinas have significantly elevated cAMP levels has raised the possibility that mislocalized class I mutants cause cell death by inappropriate activation of signaling pathways (Weiss *et al.,* 1995). Alternatively, cell death may be triggered by altered trafficking of other phototransduction proteins that are normally cotransported with rhodopsin to the OS.

A study of photoreceptor degeneration in chimeric mice derived from the fusion of P347S transgenic and wild-type embryos indicates that the mechanism of class I rhodopsin-induced cell death may be more complex (Huang *et al.,* 1993). In such chimeric mouse retinas, which contain both normal and transgenic photoreceptors distributed in patches, photoreceptor degeneration is spatially uniform, affecting both normal and transgenic photoreceptors. This implies that the pathways leading to photoreceptor cell death are not completely cell autonomous and suggests a role for cell–cell interactions, perhaps via trophic factors. In addition, this finding may shed light on the mechanisms of cone photoreceptor death caused by mutations in rod-specific genes.

B. Are Rhodopsin Transport Proteins Candidates for Causing Retinal Degenerative Diseases?

The study of inherited retinal degenerations has benefited enormously from a candidate gene approach, as opposed to a positional cloning approach, to identifying novel disease-causing genes (reviewed in Dryja and Berson, 1995). In this approach, a gene is selected, on the basis of its known function, as a reasonable candidate for causing retinal degeneration. A large sample of unrelated individuals with inherited retinal degenerative disease is then screened for mutations in the candidate gene. Mutations identified in this screen are then subjected to clinical and genetic analysis of the relevant pedigrees to determine whether they cosegregate with the disease in question.

If defective protein targeting in photoreceptors leads to cell death, then genes required for proper rhodopsin localization are good candidates for hereditary retinal degenerative diseases. At first glance, this may seem unlikely because many of the proteins that have been proposed to be involved in rhodopsin transport, such as Rab6 and Tctex-1, are widely expressed in many tissues. Although most of the genes that have been associated with RP to date are photoreceptor specific, this is likely to be due in part to the selection bias inherent to the candidate gene approach.

Moreover, mutations in several widely expressed genes are known to result in remarkably ocular-specific pathology. For instance, mutations in the enzyme ornithine aminotransferase cause gyrate atrophy (Mitchell *et al.,* 1988; Ramesh *et al.,* 1988), and mutations in the retinitis pigmentosa GTPase regulator (RPGR) are found in some cases of X-linked RP (Meindl *et al.,* 1996). Indeed, *TCTEL1,* the human homologue of *tctex-1,* has already been proposed as a candidate gene for retinal cone dystrophy 1 on the basis of their colocalization to 6q25.2–q25.3 (Watanabe *et al.,* 1996). It will be very interesting indeed to find out whether mutations in Tctex-1 or other cellular components involved in photoreceptor protein trafficking lead to photoreceptor degeneration.

VIII. Concluding Remarks

This review has sought to summarize our progress in the understanding of rhodopsin trafficking in the photoreceptor (Fig. 5) and its relationship with some forms of inherited retinal dystrophy. Several cellular components potentially involved in rhodopsin transport have been identified. Mutations in these components could conceivably result in photoreceptor degeneration. An important first step will be to determine whether conditional ''knockout'' or dominant-negative inhibition of these molecules leads to rhodopsin mistargeting and/or retinal degeneration. Elucidating the mechanisms that determine the polarized expression of rhodopsin will be vital if we are to understand the trafficking of other important photoreceptor proteins. Furthermore, it is conceivable that our progress in understanding polarity and function in the photoreceptor will prove to be useful in the study of ciliated olfactory and auditory neurons, and possibly of other cell types as well. Finally, a more complete comprehension of the pathways by which rhodopsin misfolding and/or mislocalization lead to cell death will allow us to make substantial headway toward the eventual goal of designing rational therapeutic interventions for patients with RP.

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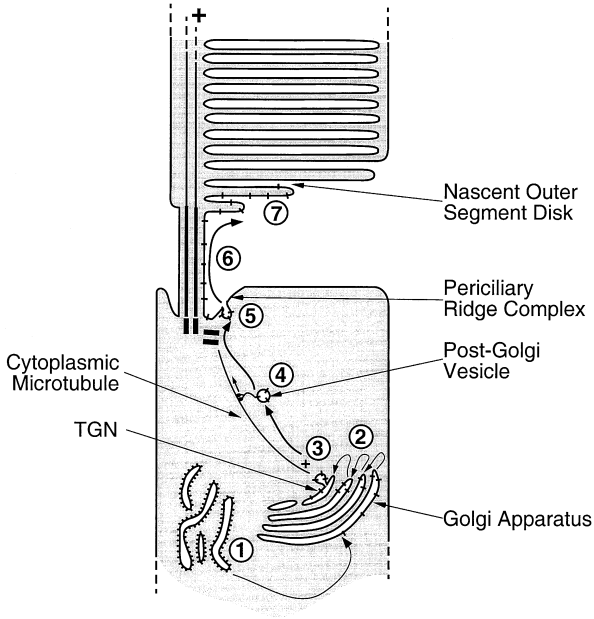

FIG. 5 Model of rhodopsin transport from IS to OS. This figure summarizes a proposed sequence of transport steps that rhodopsin may follow from its synthesis at the ER to its incorporation into OS disk membranes. (1) Synthesis, cotranslational insertion, and core N-glycosylation of rhodopsin on rough endoplasmic reticulum membranes. Folding of rhodopsin into its native confirmation is also likely to occur at the ER. (2) Modification of N-linked oligosaccharides during the transit of rhodopsin through the Golgi apparatus. (3) Sorting into and budding of OS-destined post-Golgi vesicles at the TGN; Rab proteins may be involved. (4) Transport of rhodopsin-bearing post-Golgi vesicles by cytoplasmic dynein along IS microtubules to the apical IS. (5) Release of rhodopsin-bearing vesicles from microtubules and fusion with the periciliary ridge complex plasma membrane. (6) Translocation of rhodopsin from the IS to the OS. (7) Incorporation of rhodopsin into nascent OS disks.

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Calcium Signaling during Abiotic Stress in Plants

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Plants experience a wide array of environmental stimuli, not all of which are favorable, and, unlike animals, are unable to move away from stressful environments. They therefore require a mechanism with which to recognize and respond to abiotic stresses of many different types. Frequently this mechanism involves intracellular calcium. Stress-induced changes in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$) occur as a result of influx of Ca^{2+} from outside the cell, or release of Ca^{2+} from intracellular stores. These alterations in $[Ca^{2+}]_{cyt}$ constitute a signal that is transduced via calmodulin, calcium-dependent protein kinases, and other Ca^{2+}-controlled proteins to effect a wide array of downstream responses involved in the protection of the plant and adjustment to the new environmental conditions. Ca^{2+} signaling has been implicated in plant responses to a number of abiotic stresses including low temperature, osmotic stress, heat, oxidative stress, anoxia, and mechanical perturbation, which are reviewed in this article.
KEY WORDS: Calcium, Cell signaling, Plants, Abiotic stress, Low temperature, Aequorin.

I. Introduction

Changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) control numerous processes in plants, animals, and yeast (Bush, 1995; Campbell, 1983). Ca^{2+} is a cytotoxic ion, and its levels are tightly regulated in eukaryotic cells, the basal concentration in the plant cytosol being of the order of 100–200 n*M*. On stimulation, an influx of Ca^{2+} into the cytosol occurs via Ca^{2+}-selective ion channels either in the plasma membrane or in organelles (Piñeros and

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Tester, 1997; White, 1998). As a result, $[Ca^{2+}]_{cyt}$ is elevated transiently and mediates an array of downstream processes through Ca^{2+}-dependent proteins such as calmodulin (Zielinsky, 1998), Ca^{2+}- and calmodulin-dependent protein kinases (CDPKs) (Poovaiah *et al.,* 1997), and phosphatases (Luan, 1998; Sopory and Munshi, 1998). Localized $[Ca^{2+}]_i$ elevations may also occur in specific organelles such as the chloroplast (Johnson *et al.,* 1995), and these are likely to control events specific to the organelle. Ca^{2+} homeostasis is restored by removal of Ca^{2+} by pumps or antiporters that bring the $[Ca^{2+}]_{cyt}$ back to basal levels. (For reviews detailing the mechanisms used by plants to regulate calcium homeostasis, see Bush, 1995; Muto, 1992; Poovaiah and Reddy, 1993; Trewavas and Malhó, 1998.)

Calcium has been implicated in a number of abiotic stress signaling pathways with varying degrees of directness. Some of the various approaches for identifying Ca^{2+} as a signaling component in a response are illustrated in the following section describing low temperature responses. Cold signaling will be discussed in most detail, as an illustration of the types of techniques used to elucidate environmental stress Ca^{2+} signaling. Some of the earliest work at the whole plant level involved application of exogenous Ca^{2+} and resultant mediation of protective responses, this type of data emerging years before direct demonstration of a stress-induced increase in $[Ca^{2+}]_{cyt}$ was possible. Such data are difficult to interpret, and it is not possible to discount the possibility that Ca^{2+} has exerted a nutritional effect or causes the response through imposing osmotic stress. However, more recently, direct measurement of abiotic stress-induced $[Ca^{2+}]_{cyt}$ elevations, their correlation with downstream end responses, and the use of Ca^{2+} homeostasis inhibitors have strengthened previous claims. It has been suggested that in order to show Ca^{2+} is involved in a signaling pathway linking an end response to a stimulus, the following evidence is required: (1) a measurement of the rise in $[Ca^{2+}]_{cyt}$ in response to the stimulus is needed; (2) an artificial $[Ca^{2+}]_{cyt}$ elevation of equal or lower magnitude to this should be able to activate the end response; and (3) it should be possible to block or lower the end response by inhibiting the natural rise in $[Ca^{2+}]_{cyt}$ in response to the stimulus (Jaffe, 1980). As the reader will be aware, not all of these criteria can be met in all of the studies described here, and, in particular, artificial elevations of $[Ca^{2+}]_{cyt}$ are often difficult to elicit in plant systems. Also, it is often the case that Ca^{2+} is necessary to achieve the response but not sufficient. In such cases, the second of the criteria above cannot be fulfilled. However, in combination the data described in this chapter provide a large body of evidence for a role for Ca^{2+} in abiotic stress signaling.

It should be noted that the separation of abiotic stress into the seven broad categories used here should not be considered at all inviolate, as there is much overlap. For instance, salinity involves an osmotic stress

component (Niu *et al.,* 1995), mechanical, cold, and drought stress (Moran *et al.,* 1994; Prasad, 1996; Yahraus *et al.,* 1995) all cause oxidative stress, and freezing temperatures cause dehydration stress (Thomashow, 1994). The question of overlap of stresses and their responses, cross talk between pathways, and specificity will be discussed at the end of the chapter.

II. Low Temperature Stress

A. Cold Sensitivity and Freezing Sensitivity

Plants encounter variations in temperature in all environments. During exposure to low temperature, plants may experience chilling injury (at above zero temperatures) and/or freezing injury (at subzero temperatures). Chilling stress is experienced by so-called chilling-sensitive plants, particularly tropical and subtropical species (Lyons, 1973). Damage is caused in chilling-sensitive species when the temperature falls below approximately 10°C and ranges from loss of vigor to death (Thomashow, 1994). Changes in membrane properties and solute leakage from cells are among the observed effects of chilling (Prasad *et al.,* 1994a; Thomashow, 1994). The temperature at which chilling injury is experienced depends on the type of plant and the normal growth temperature of that plant.

Freezing injury occurs in plants at subzero temperatures and appears to be largely a result of dehydration which occurs during freezing and subsequent thawing (Thomashow, 1994). Ice forms in the extracellular spaces and causes water to move from inside the cell to outside. This water returns to the inside of the cell on thawing (Thomashow, 1994). The large influx and efflux of water to and from the cell during thawing and freezing puts a strain on the plasma membrane, ultimately causing it to be damaged and resulting what is known as ''expansion-induced cell lysis.'' The plasma membrane, therefore, is considered to be the primary site of freezing injury (Steponkus, 1984).

Plants can acclimate to chilling (Prasad *et al.,* 1994a) or freezing (Levitt, 1980) temperatures during exposure to less severe temperatures and, in this way, increase their cold temperature or freezing temperature tolerance, often by several degrees. Obviously this has a huge potential for influencing the survivability of plants during cold weather. Some chill-tolerant species are capable of cold acclimation, and these become more frost tolerant after treatment with positive temperatures in the range of 2°–6°C; however, not all chilling-tolerant plants are capable of cold acclimation (Hughes and Dunn, 1996).

During cold acclimation to freezing temperatures, a number of changes occur including alteration of membrane lipid composition, accumulation of sugars, proline, soluble proteins, and organic acids, and the expression of many genes (Hughes and Dunn, 1996; Thomashow, 1998). The changes that occur in the phospholipid composition of the plasma membrane alter the way in which the membrane behaves during cold temperature, rendering cells of cold acclimated plants less affected by expansion-induced lysis during thawing (Uemura *et al.,* 1995).

There is increasing evidence that calcium is involved in acclimation to chilling and freezing temperatures (Monroy and Dhindsa, 1995; Monroy *et al.,* 1993), making the study of the initial Ca^{2+} signaling events during cold treatment of particular interest.

B. Cold-Induced $[Ca^{2+}]_{cyt}$ Elevation

1. Subcellular Origin and Characterization of the Ca^{2+} Ion Signal

A reduction in temperature causes an instantaneous influx of Ca^{2+} ions into the cell and consequent rise in $[Ca^{2+}]_i$. The link between cold treatment and Ca^{2+} influx has been observed for some time. Increased influx of radiolabeled Ca^{2+} ($^{45}Ca^{2+}$) into roots was observed in winter wheat treated at 2°C at high Ca^{2+} concentrations (Erlandson and Jensen, 1989), and similar observations have been made in maize roots (Rincon and Hanson, 1986). The cold-induced influx of Ca^{2+} was found to be due to the opening of Ca^{2+} channels rather than a general, cold-induced increased membrane permeability, and this has been demonstrated by the use of specific pharmacological agents. Influx of $^{45}Ca^{2+}$ into cold-shocked maize roots was strongly inhibited by the Ca^{2+} channel blocker lanthanum, though only poorly by verapamil (Rincon and Hanson, 1986). Other workers also reported the inhibition of cold-induced Ca^{2+} influx in maize roots by calcium channel blockers (De Nisi and Zocchi, 1996), with strong inhibition by verapamil and nifedipine, with this particular study suggesting that voltage-gated Ca^{2+} channels were involved. Reducing the temperature from 25° to 4°C caused a large increase in the influx of radiolabeled calcium ($^{45}Ca^{2+}$) in alfalfa protoplasts (Monroy and Dhindsa, 1995). The Ca^{2+} channel blockers verapamil, lanthanum, and nitrendipine or the Ca^{2+} chelator BAPTA inhibited calcium influx (Monroy and Dhindsa, 1995). The exact nature of the cold-sensitive calcium channel(s) still remains unclear.

The cold-induced Ca^{2+} influx via Ca^{2+} channels results in an immediate transient elevation in $[Ca^{2+}]_{cyt}$. This was first measured in intact whole plants expressing the recombinant Ca^{2+} reporter protein, aequorin (Knight

et al., 1991). Cold-induced $[Ca^{2+}]_{cyt}$ elevations have been measured in tobacco (Knight *et al.,* 1991, 1992, 1993), *Arabidopsis* (Knight *et al.,* 1996; Lewis *et al.,* 1997; Polisensky and Braam, 1996), and the moss *Physcomitrella patens* (Russell *et al.,* 1996) using the recombinant aequorin method. The magnitude of the $[Ca^{2+}]_{cyt}$ elevation was markedly reduced by the Ca^{2+} channel blocker lanthanum (Knight *et al.,* 1996; Knight *et al.,* 1992), again indicating that it is due, at least in part, to the influx of Ca^{2+} via plasma membrane Ca^{2+} channels. Ding and Pickard (1993a) identified a plasmalemma mechanosensitive Ca^{2+} channel, the activity of which was increased by a reduction in temperature, suggesting the involvement of this channel in the cold-induced elevation of $[Ca^{2+}]_{cyt}$. The authors speculated that this channel could be involved in the sensing of low temperature. Work using protoplasts isolated from aequorin-expressing tobacco demonstrated that the magnitude of the cold-induced $[Ca^{2+}]_{cyt}$ elevation was dependent on the organization of the cytoskeleton, with disruption of either the microtubule or microfilament networks stimulating the cold-induced response (Mazars *et al.,* 1997).

Work using aequorin has indicated that in addition to the influx of external Ca^{2+} there is also a release of Ca^{2+} from the vacuole, possibly via IP_3-sensitive channels (Knight *et al.,* 1996). *Arabidopsis* plants expressing recombinant aequorin targeted to the cytosolic face of the tonoplast membrane (an area we have termed the vacuolar microdomain) were used to demonstrate the $[Ca^{2+}]_{cyt}$ elevations occurring in this locale of the cell. Comparison of the cold-induced Ca^{2+} kinetics in this area with those throughout the cytosol (measured using aequorin targeted to the cytosol) suggested the involvement of vacuolar calcium in the cold shock response. Whether the influx component and the vacuolar release component of the $[Ca^{2+}]_{cyt}$ elevation have different roles is not yet known. However, if different subcellular locations were to exist for the downstream components of calcium signaling, it would be possible that the $[Ca^{2+}]_{cyt}$ elevations around different organelles could trigger different effector proteins and thus separate cold-induced downstream responses. The inhibition of the $[Ca^{2+}]$ elevation in the vacuolar microdomain by lithium chloride and neomycin, both inhibitors of phosphatidylinositol cycling, suggested that inositol trisphosphate (IP_3) may be involved in mediating this release of Ca^{2+} (Knight *et al.,* 1996). It has also been shown in maize roots that cold-induced phosphatidylinositol breakdown was inhibited by application of calcium channel blockers (De Nisi and Zocchi, 1996). The second messenger IP_3 is one of the breakdown products of phosphatidylinositol; therefore, these data suggested that the cold-induced calcium elevation mobilizes IP_3 as part of the cold signal transduction pathway, indicating a role for IP_3 (De Nisi and Zocchi, 1996). Data reporting the accumulation of IP_3 in response to cold support this hypothesis (Smolenska Sym and Kacperska, 1996).

When compared in seedlings of the same age, the cold shock responses of tobacco and *Arabidopsis* were found to be similar but the Ca^{2+} transient was slightly more prolonged in tobacco (Knight *et al.,* 1996). It is possible that this reflects the difference in cold sensitivity between the two species: *Arabidopsis* is a chilling-tolerant species, whereas tobacco is cold sensitive. The cold-induced $[Ca^{2+}]_{cyt}$ was found to be attenuated in both species when repeated 3 min after the first stimulation, recovering partially after 10 min. Thirty minutes after the initial cold stimulation, a further stimulation produced a normal recovered response in tobacco but not in *Arabidopsis,* indicating there was still some ''memory'' of the stimulus in *Arabidopsis* (Knight *et al.,* 1996). This difference was observed in the longer term also. Cold-induced $[Ca^{2+}]_{cyt}$ elevations were measured in seedlings treated at 4°C for 3 hr per day on three consecutive days and allowed to recover overnight. In *Arabidopsis,* the cold pretreated plants responded with a slightly smaller but more prolonged $[Ca^{2+}]_{cyt}$ elevation, indicating that some information relating to the previous stimulation was still retained. However, this was not observed in tobacco, indicating that the cold-sensitive plant did not retain this information (Knight *et al.,* 1996). It is possible that these differences may have some bearing on the ability (or lack of) to cold acclimate (Knight *et al.,* 1996). It is possible that an alteration in the cold ''calcium signature'' (or the precise kinetics of the $[Ca^{2+}]_{cyt}$ elevation) reflects an alteration in Ca^{2+} homeostasis occurring during acclimation and controls subsequent altered responses such as gene expression in acclimated plants.

2. Spatiotemporal Characterization

Advances in luminescence measurement technology have meant that stress-induced $[Ca^{2+}]_{cyt}$ elevations can be monitored spatially in different organs of the plant by aequorin luminescence imaging. Gradual cooling of tobacco (Campbell *et al.,* 1996) has shown a differential sensitivity of the root and the aerial parts of the plant. Cooling from 25°C to 17°–18°C elicited $[Ca^{2+}]_{cyt}$ elevations in the roots of tobacco. When plants were cooled further to 10°C, $[Ca^{2+}]_{cyt}$ elevations were seen in the cotyledons (Campbell *et al.,* 1996). As the temperature was reduced to 2°C, the roots stopped responding but the cotyledons continued (Campbell *et al.,* 1996). Similar behavior has been observed in *Arabidopsis* (Fig. 1A; see color insert). In the case of *Arabidopsis* or tobacco, roots respond at higher temperatures and the aerial parts at lower temperatures, as might be expected from considering the range of temperatures each would normally experience. In *Arabidopsis* we have observed that when seedlings are cooled gradually, the resulting Ca^{2+}-dependent luminescence response shows a bimodal form, with a first and second peak of aequorin luminescence occurring at a higher and later a

lower temperature (Fig. 1B; see color insert). When the roots of a mature tobacco plant were cooled while the leaves were thermally isolated, a $[Ca^{2+}]_{cyt}$ elevation was detected in some of the leaf petioles, and was later seen throughout the leaves. This occurred after a lag time of 3 min, suggesting that a signal may have been transmitted between tissues, and the form of this signal has been speculated as being hydraulic (Campbell *et al.,* 1996).

3. Ca^{2+} as a Mechanism for Cold Sensing?

The $[Ca^{2+}]_{cyt}$ elevations described above occur very rapidly after cold stimulation, and therefore it would seem possible that calcium entry into the cytosol constitutes the primary cold sensing mechanism in plants. Sensory transduction is often mediated via ion channels (Pickard and Minchin, 1990), and it has been suggested by some authors that the primary sensor is a plasma membrane calcium channel that the primary (Ding and Pickard, 1993a; Minorsky, 1989). Minorsky (1989) remarked that many of the physiological processes affected by rapid cooling (defined by the author to be within the range of 1°–10°C min^{-1}) are also known to be Ca^{2+} dependent; therefore, rapid cooling could be associated with graded increases in $[Ca^{2+}]_{cyt}$ that act as a sensory transduction system. Evidence exists suggesting that Ca^{2+} signaling is necessary for the initial perception of cold. Rapid chilling quickly interrupts phloem translocation, and the interruption is abolished by lanthanum (Pickard and Minchin, 1990). In the longer term, lanthanum inhibition of a cold-induced $[Ca^{2+}]_{cyt}$ transient was shown to correlate with inhibition of cold-induced *KIN1* expression after 1 hr (Knight *et al.,* 1996), and the cold-induced Ca^{2+} influx has been shown to be necessary for the cellular changes that accompany cold acclimation (see Section II.C) (Monroy and Dhindsa, 1995; Monroy *et al.,* 1993).

For a number of years it was known that membrane depolarizations occur in whole plant tissues in response to cold (Minorsky and Spanswick, 1989). In 1989, Minorsky and Spanswick published intracellular microelectrode measurements of membrane depolarizations in single cortical cells of cucumber roots in response to rapid cooling and discovered that, on a single cell level, the depolarizations were graded according to the rate and amplitude of the temperature drop. These single cell measurements eliminated the possibility that the graded responses observed were due to different numbers of cells responding in whole tissues and thus confirmed that individual cells were responding in a truly graded manner (Minorsky and Spanswick, 1989). This type of graded response (in contrast to ''all or nothing'' responses) is considered to be typical of sensory processes and suggested to the authors that plant cells possess a cold sensing mechanism. The calcium channel blocker lanthanum strongly inhibited these depolarizations, indicating a role for calcium in this sensory process. EGTA also gave

a small but significant inhibition of the response; however, preliminary studies indicated that the Ca^{2+} channel blockers diltiazem, verapamil, and nifedipine had no effect (Minorsky and Spanswick, 1989). The authors accounted for the range of effects by the fact that there are many classes of Ca^{2+} channel blockers, not all of which inhibit any one particular channel. The amplitudes of rapid cooling-induced electrical responses were enhanced by Ca^{2+} added to the external medium, again implicating calcium in the sensory transduction process (Minorsky and Spanswick, 1989).

More recently, cold-induced membrane depolarizations and $[Ca^{2+}]_{cyt}$ elevations have been measured in parallel in *Arabidopsis* (Lewis *et al.,* 1997) in order to investigate the relationship between the two events. The $[Ca^{2+}]_{cyt}$ elevation measured by the recombinant aequorin method appeared to occur almost simultaneously with the depolarization, and within the time resolution achievable in these experiments, it was not possible to ascertain which event occurred first (Lewis *et al.,* 1997). After treatment with the anion channel inhibitor NPBB, the cold shock-induced depolarization was reduced but the Ca^{2+} elevation was unaffected. These data help in understanding the order of depolarization and Ca^{2+} elevation events. The results suggest that cold shock first activates Ca^{2+}-permeable channels in the plasma membrane, resulting in an inward current that depolarizes the plasma membrane and elevates $[Ca^{2+}]_{cyt}$. As $[Ca^{2+}]_{cyt}$ reaches micromolar levels, anion channel activity increases (Lewis *et al.,* 1997) and prolongs the depolarization. Although the anion channel activity may be important in continuing the depolarization, it appears from the data that it is not responsible for the initial depolarization event. Together, these data indicate that Ca^{2+} is a key part of the sensory process in cold signal transduction.

C. Demonstration of the Role of Ca^{2+} and Other Ca^{2+} Signaling Components in Cold Hardiness, Cold Acclimation, and Cold-Induced Gene Expression

1. Calcium

Calcium is a cytotoxic ion, and therefore prolonged elevated cellular concentrations of Ca^{2+} would be detrimental to the cell. It has been argued that one of the chief causes of cold damage is due to the cytotoxicity of cold-induced high Ca^{2+} levels (Minorsky, 1985; Woods *et al.,* 1984a,b), although this view is less popular now. It seems likely that, through evolution, plants have ''made use'' of the pathological effect of the entry of a toxic ion into cells, and have used this as the basis for a signaling system. It could be argued that as Ca^{2+} appears to be used as a sensing system for cold (see Section II,B,3 above), elevations in $[Ca^{2+}]_{cyt}$ levels might bring

about benefits which could outweigh the disadvantages. It would be expected, therefore, that elevations in $[Ca^{2+}]_{cyt}$ would trigger at least some of the mechanisms of cold tolerance and cold acclimation. The beneficial effects of Ca^{2+} on the response of plants to cold stress have been observed and demonstrated at the whole plant and the cellular level.

Calcium has been implicated in the process of cold hardening in studies such as one in which cold-hardened rice seedlings germinated in the presence of calcium chloride recovered from chilling stress more effectively than did normal cold-hardened seedlings (Mei-ru *et al.,* 1996). Calcium treatment also increased the levels of glutathione and ascorbic acid in treated plants, both of which are stress protectants (Mei-ru *et al.,* 1996). Exogenous application of calcium resulted in increased Ca^{2+} concentrations in leaves and fruits of apple and pear, and improved cold hardiness (Raese, 1996). A number of treatments to cucumber roots, including the application of calcium nitrate, have been shown to cause significant increases in chilling tolerance, as assessed by ability of the roots to elongate (Jennings and Saltveit, 1994). It appears from studies of this type that Ca^{2+} is able to confer some positive benefits during cold stress, though it cannot be assumed that these are all brought about by calcium as a second messenger.

Evidence for the role of Ca^{2+} as a second messenger mediating benefits during cold stress has been obtained by measuring the Ca^{2+} dependency of a number of end responses. Proline accumulation is one of the responses elicited by a number of stresses including exposure to low temperature. Proline accumulation correlates with improved tolerance of osmotic stress and temperature extremes, and a number of mechanisms have been proposed whereby this may occur (Verbruggen *et al.,* 1993). The necessity of calcium for cold-induced accumulation of proline in *Amaranthus* (Bhattacharjee and Mukherjee, 1995) and in tomato seedlings and cell cultures (De *et al.,* 1996) was demonstrated by the use of inhibitors of calcium signaling. In each case, EGTA and lanthanum inhibited the cold-induced increase in proline levels, whereas the presence of calcium chloride increased cold-induced proline levels.

The nonprotein amino acid γ-aminobutyric acid (GABA) is synthesized within minutes of stress stimulation and, like proline, appears in response to diverse environmental stresses although its role is not clear. Experiments carried out using asparagus cells showed that the cold shock-induced Ca^{2+} elevation controlled the activity of L-glutamate decarboxylase (GAD), the enzyme which synthesizes GABA (Cholewa *et al.,* 1997). Plant GAD is a Ca^{2+}-binding protein (Baurn *et al.,* 1993) and its activity is stimulated by Ca^{2+}/calmodulin (Arazi *et al.,* 1995; Snedden *et al.,* 1996). Synthesis of GABA was also induced by artificially elevating $[Ca^{2+}]_{cyt}$ (Cholewa *et al.,* 1997).

During cold stress a battery of genes are induced (for examples, see Cattivelli and Bartels, 1990; Jarillo *et al.,* 1993; Kurkela and Franck, 1990; Thomashow, 1994; White *et al.,* 1994). Many of the genes induced are also induced by other stresses, particularly drought (Nordin *et al.,* 1991) and the plant hormone abscisic acid (ABA) (Mäntylä *et al.,* 1995). In *Arabidopsis* most of the cold-induced genes described respond to drought and/or ABA also (Hughes and Dunn, 1996). There are exceptions, however, including the genes *RCI1* and *RCI2* which are induced only by cold and not by ABA or water stress (Jarillo *et al.,* 1994). Cold-induced genes requiring Ca^{2+} for their full expression have been described in a number of species. They include the *MLIP15* gene of maize (Berberich and Kusano, 1997), *CAS15* and *CAS18* in alfalfa (Monroy and Dhindsa, 1995), and *TCH3* and *KIN1* in *Arabidopsis* (Knight *et al.,* 1996; Polisensky and Braam, 1996; Tähtiharju *et al.,* 1997).

Over recent years evidence has emerged that Ca^{2+} influx is a key factor in controlling a plant's ability to cold acclimate. Using cell suspension cultures of a freezing-tolerant cultivar of alfalfa, the effects of modifying Ca^{2+} homeostasis have been studied (Monroy *et al.,* 1993). Freezing tolerance was increased by cold acclimation at 4°C, but this effect was greatly diminished by the inclusion of EGTA and abolished by the calcium channel blockers lanthanum and verapamil (Monroy *et al.,* 1993). Use of the same inhibitors demonstrated that Ca^{2+} was also involved in the phosphorylation of preexisting protein (Monroy *et al.,* 1993). The calcium channel blocker lanthanum also inhibited the cold induction of three acclimation-specific cDNA clones (Monroy *et al.,* 1993).

Further investigation into the relationship between Ca^{2+} influx and cold acclimation in alfalfa cells was performed using radiolabeled calcium ($^{45}Ca^{2+}$) in protoplasts (described earlier in Section II,B,1) and monitoring the expression of two cold acclimation-specific genes, *CAS15* and *CAS18* (Monroy and Dhindsa, 1995). Reducing the temperature from 25°C to 4°C caused a large increase in the influx of calcium and also induced expression of the two acclimation-specific genes (Monroy and Dhindsa, 1995). The calcium channel blockers verapamil, lanthanum, and nitrendipine or the Ca^{2+} chelator BAPTA inhibited both Ca^{2+} influx and gene expression, suggesting that expression of these genes is regulated by an influx of Ca^{2+} via a specific subset of Ca^{2+} channels. The level of inhibition achieved with the channel blockers varied depending on which was used, suggesting the response depends on a specific subset of Ca^{2+} channels. In addition, the application of the Ca^{2+} ionophore A23187 or Ca^{2+} channel agonist BAY K8644 at 25°C caused Ca^{2+} influx and expression of the genes (Monroy and Dhindsa, 1995). Maize leaf protoplasts expressing a chimeric gene construct consisting of a fusion of the cold- and ABA-inducible promoter *HVA1* with a synthetic green fluorescent protein (GFP) reporter gene

were used to demonstrate the involvement of signaling components in the expression of this gene (Sheen, 1996). Treatment with Ca^{2+} ionophores resulted in increased expression of the gene, in the absence of cold or ABA, indicating that Ca^{2+} is involved in regulation of *HVA1* gene expression (Sheen, 1996).

In whole plant studies, induction of the *Arabidopsis* gene *KIN1* in response to cold shock was strongly inhibited by the presence of lanthanum or EGTA (Knight *et al.,* 1996). Further work with *KIN1* and the closely related cold-induced gene *KIN2* (also known as *COR6.6*) confirmed that both genes were calcium regulated, although possibly to different extents (Tähtiharju *et al.,* 1997). The Ca^{2+} regulation of *KIN2* expression has also been demonstrated in single cells by a microinjection approach (Wu *et al.,* 1997). This work is described more fully in Section II,D, concerning the role of ABA in cold and drought responses. EGTA did not significantly reduce cold-induced *KIN2* transcript levels (Tähtiharju *et al.,* 1997). The Ca^{2+} channel inhibitor ruthenium red caused both an inhibition of gene expression and a small loss of cold acclimation-associated freezing tolerance, whereas lanthanum, gadolinium, and EGTA all abolished cold acclimation-acquired gains in freezing tolerance, implicating Ca^{2+} in this process (Tähtiharju *et al.,* 1997).

Together these data demonstrate the role of Ca^{2+} in initiating cold gene expression and mediating cold acclimation.

2. Calmodulin, Ca^{2+}-Binding Proteins, and Other Ca^{2+}-Regulated Components

A number of mechanisms exist that may link cold-induced Ca^{2+} elevations to downstream effects. To mediate the effects of Ca^{2+}, effector proteins that interact with Ca^{2+} are required. Downstream effects can be mediated directly via Ca^{2+}-dependent (de)phosphorylation events (Minorsky, 1989) or transduced via Ca^{2+}-binding proteins such as calmodulin. Calmodulin transcript levels have been shown to alter in response to certain stresses (Braam and Davis, 1990), indicating that calmodulin may be involved in the modulation of stress responses. Expression of the *TCH* genes which encode calmodulin-related proteins has been shown to be induced by cold, also suggesting that calmodulin (or similar Ca^{2+}-binding proteins) may be important during cold signal transduction (Polisensky and Braam, 1996). In alfalfa, differential regulation of calmodulin transcript levels was not seen but CDPK sequences were upregulated by low temperature (Monroy and Dhindsa, 1995). The role of the Ca^{2+} modulating protein calmodulin in cold signal transduction has been indicated in several studies. In the studies described above, the cold-mediated induction of several genes and the phosphorylation of proteins seen in alfalfa was reduced by inhibitors

of calmodulin (Monroy *et al.,* 1993). W7, an antagonist of calmodulin and CPDKs, also inhibited the development of freezing tolerance in alfalfa, indicating the involvement of calmodulin and/or CDPK(s) in the cold acclimation process in these cells (Monroy *et al.,* 1993). Similarly, W7 also inhibited cold acclimation and acquisition of freezing tolerance in whole *Arabidopsis* plants (Tähtiharju *et al.,* 1997). Using a GFP reporter construct to measure expression of the cold-regulated gene *HVA1,* it was observed that expression of the *Arabidopsis* CDPK1 enhanced ABA-induced *HVA1* expression (Sheen, 1996). These results suggested that CDPK1 is involved in ABA-induced expression of the *HVA1* gene, and that CDPKs might also participate in the cold-induced expression of this gene (Sheen, 1996).

During the sequence of changes in membrane potential occurring during cold stimulation (Section II,B,3), the initial depolarization is followed by a repolarization phase in which the membrane potential returns to normal resting levels. The calmodulin antagonists TFP, 48/80, and W7 all caused a marked slowing of the repolarization phase of the rapid cooling response, indicating the involvement of calmodulin in returning the membrane potential to normal resting levels, possibly by mediating channel closure (Minorsky and Spanswick, 1989).

Cold-induced protein phosphorylation has been reported in alfalfa (Monroy *et al.,* 1993), and in maize roots cold shock enhanced the phosphorylation of specific membrane proteins (Zocchi *et al.,* 1983). Cold acclimation-specific Ca^{2+} dependent protein kinases (CDPKs) have now been identified in *Arabidopsis,* and these have been implicated in mediating cold acclimation (Tähtiharju *et al.,* 1997).

The activity of other proteins may be controlled by Ca^{2+}, and these may have beneficial effects during cold or freezing treatment. The green alga *Duniella salina* possesses a cold-regulated acyl hydrolase active in microsomal preparations, the activity of which is increased by the presence of Ca^{2+} (Norman and Thompson, 1986). The workers who identified this suggested that the retailoring of membrane phospholipids which occurs in response to chilling in *Duniella* cells is due to the cold-regulated activity of this enzyme (Norman and Thompson, 1986). Similarly, phospholipase A activity has been observed in membranes of the protozoan *Tetrahymena* during acclimation to low temperature, and this may also have a function in adaptation to chilling temperatures (Thompson, 1986) as membrane fluidity is correlated with lipid composition.

D. The Role of Abscisic Acid in Relation to Cold- and Drought-Induced Ca^{2+} Signaling

The hormone abscisic acid (ABA) is involved in many physiological processes including both drought responses and the induction of cold

hardiness through cold acclimation. Increased levels of ABA in plant tissues occur in response to low temperature treatment (Chen *et al.,* 1983; Daie and Campbell, 1981; Kacperska-Palacz, 1978; Mohapatra *et al.,* 1987) and in response to drought (Cronish and Zeevaart, 1984; Plant *et al.,* 1991), and thus the following section relates to both of these stresses. Many of the genes that are induced by cold can be induced also by ABA (Hughes and Dunn, 1996), and in *Arabidopsis* common polypeptides have been found to accumulate in response to either cold or ABA treatment, indicating a common pathway (Lång *et al.,* 1989). However, not all of the cold-induced polypeptides were found in ABA-treated plants, suggesting that more than one pathway exists (Lång *et al.,* 1989). Application of exogenous ABA has been shown to increase cold hardiness in a number of species including *Arabidopsis* (Lång *et al.,* 1989), and the ABA biosynthesis inhibitor fluridone has been shown to prevent cold acclimation in cell cultures of *Solanum commersonii* (Zhu *et al.,* 1993). These data support the hypothesis that ABA accumulation allows the plants to become cold acclimated (Chen *et al.,* 1983), an idea further strengthened by reports on the effects of ABA on ABA mutants. The ABA-deficient mutant *aba-1* was unable to cold acclimate to the same levels as wild-type plants (Heino *et al.,* 1990), and the ABA-insensitive mutant *abi1* was impaired or slower in its ability to cold acclimate (Gilmour and Thomashow, 1991; Lång *et al.,* 1994). Work with these mutants has, in addition, demonstrated that some cold-induced genes require ABA for their expression whereas others do not, suggesting a number of different pathways operating (Mäntylä *et al.,* 1995). The endogenous levels of ABA accumulating in response to low temperature treatment of *Arabidopsis* did not correlate with increases in freezing tolerance; however, it was suggested that a transient accumulation of ABA preceding freezing tolerance may be the trigger for cold acclimation (Lång *et al.,* 1994).

The interaction between Ca^{2+} and ABA appears to be complex, and it is possible that both Ca^{2+}-dependent and Ca^{2+}-independent ABA-inducible signaling pathways may operate. ABA can elevate $[Ca^{2+}]_{cyt}$ (Curvetto *et al.,* 1994; McAinsh *et al.,* 1992), and therefore it could be envisaged that cold-induced ABA accumulation could be responsible for eliciting the $[Ca^{2+}]_{cyt}$ elevation which is observed in response to low temperature. However, the $[Ca^{2+}]_{cyt}$ elevation in response to low temperature is effectively instantaneous, and this time scale of events would be unlikely to allow for accumulation of ABA and its subsequent action on Ca^{2+} homeostasis. As there is a body of evidence suggesting ABA mobilizes internal Ca^{2+} release and thus is responsible for an elevation in $[Ca^{2+}]_{cyt}$, it seems likely that Ca^{2+} is involved at both an early and again at a later stage in the pathway. Indeed, the necessity for Ca^{2+} in

ABA induction of *Em* gene expression has been demonstrated in rice protoplasts (Rock and Quatrano, 1996).

The *Arabidopsis* ABA response gene *ABI1,* essential for a wide range of plant responses to ABA, encodes a protein that appears to be a Ca^{2+}-modulated phosphatase (Leung *et al.,* 1994; Meyer *et al.,* 1994). It has been suggested that ABI1, along with its homologue ABI2 (Rodriguez *et al.,* 1998), functions to integrate ABA and Ca^{2+} signals with phosphorylation-dependent response pathways (Leung *et al.,* 1994). There is, therefore, scope for cross talk between these two pathways.

Over the last few years, cyclic ADP-ribose (cADPR) has been identified as a signaling molecule that can trigger Ca^{2+} release from sea urchin microsomes and beet cell vacuoles (Allen *et al.,* 1995). cADPR mediates the response to ABA and exerts its effects via Ca^{2+} (Allen *et al.,* 1995; Wu *et al.,* 1997). Single cells of *Arabidopsis* were coinjected with GUS reporter gene constructs and signaling intermediates, and expression of *RD29-GUS* (the cold- and drought-inducible gene also known as *LTI78*) and *KIN2-GUS* (primarily a cold-inducible gene) monitored. The cold-regulated gene *KIN2* could be induced by injection of Ca^{2+} or by ABA, but not by ABA in the presence of the Ca^{2+} chelator EGTA, indicating that Ca^{2+} is required for the ABA-induced gene expression (Wu *et al.,* 1997). cADPR could substitute for Ca^{2+} or ABA in inducing the two reporter genes, and its action was also blocked by EGTA. These results suggest that one ABA signaling pathway, at least, involves cADPR effecting release of intracellular Ca^{2+} which in turn activates cold and drought gene expression (Wu *et al.,* 1997). The protein kinase inhibitors staurosporin and K252a inhibited the response of genes to Ca^{2+}, ABA, or cADPR, indicating that protein phosphorylation was required (Wu *et al.,* 1997). Injection of IP_3 was also capable of activating reporter gene expression, suggesting IP_3 too could be involved *in planta* in causing internal Ca^{2+} release and subsequent gene expression. Other data have also pointed to a role for IP_3 in cold signal transduction pathways. Both freezing temperatures and ABA have been shown to elevate levels of IP_3 in oilseed rape (Smolenska Sym and Kacperska, 1996), and the involvement of IP_3 has been implicated in the mediation of cold-induced intracellular Ca^{2+} release in other species (De Nisi and Zocchi, 1996; Knight *et al.,* 1996). Therefore IP_3 may be involved in the control of ABA-dependent gene expression. Taken together, the data gathered from these microinjection experiments indicate that IP_3 and cADPR may both participate in mediating ABA-induced gene expression, by releasing Ca^{2+} from internal stores. This elevation of $[Ca^{2+}]_{cyt}$ is followed by protein phosphorylation, which leads ultimately to gene expression. It is likely that this ABA-induced pathway is used during cold and drought induction of expression also.

III. Osmotic Stress, Drought, and Salinity Stress

A. Osmotic Stress in Plants

Much of the earth's surface is affected by drought and salinity, salinity causing problems in a substantial proportion of the world's agricultural land (Epstein *et al.,* 1980). Many of the effects of drought and salinity are similar in that they cause dehydration of the cell. However, salinity exerts an effect both as a form of osmotic stress (similar to that of drought) and through the ionic nature of sodium (Na^+). Na^+ ions interfere with the uptake of other nutrients and inhibit cellular metabolism (Pasternak, 1987; Werner and Finkelstein, 1995). In the case of both salinity and drought stress, the resultant high osmotic pressure makes it difficult for plants to absorb water. There are a number of protective cellular responses in response to salinity and drought stress, which include stomatal closure to avoid further water loss (Davies *et al.,* 1981) and accumulation of compatible solutes and other osmoprotectants (Bartels and Nelson, 1994) including proline (Savouré *et al.,* 1995). In addition to acting as an osmoprotectant (Taylor, 1996), proline has other functions including a role as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989), in protecting macromolecules against denaturation (Schobert and Tsesche, 1978), and a means of reducing the acidity of the cell (Venekamp *et al.,* 1988). Ca^{2+} has been known for some time to mediate tolerance of salinity stress (LaHaye and Epstein, 1969), and a role for Ca^{2+} as a second messenger in initiating protective responses during drought, salinity, and osmotic stress is currently emerging. Recent work now indicates that the Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin increases salinity tolerance (Pardo *et al.,* 1998). ABA plays a major role in drought responses and accumulates in response to drought (Cornish and Zeevaart, 1984; Plant *et al.,* 1991), and it regulates the expression of genes during drought stress (Bray, 1991). The possible interactions between Ca^{2+} and ABA during cold and drought signaling are discussed in Section II,D.

The fact that salinity and drought treatments cause increased expression and activity of a number of calcium signaling components suggests a role for calcium signaling in the response to these stresses (Johansson *et al.,* 1996; Perez Prat *et al.,* 1992; Pestenacz and Erdei, 1996; Urao *et al.,* 1994; Wimmers *et al.,* 1992). Salinity has been shown to increase transcript levels of genes encoding a putative endoplasmic reticulum (ER) Ca^{2+}-ATPase in tobacco cells (Perez Prat *et al.,* 1992) and a putative ER Ca^{2+}-ATPase in tomato (Wimmers *et al.,* 1992), and in *Arabidopsis* a salt-inducible gene, *AtCP1,* has been identified as encoding a Ca^{2+}-binding protein (Jang *et al.,* 1998). Calcium-dependent protein kinases (CDPKs) have been shown to

be controlled by such stresses at the levels of both transcript and protein activity. Genes encoding CDPKs that are expressed in response to drought and salt stress have been identified in *Arabidopsis* (Urao *et al.,* 1994), and expression of a CDPK in mung bean has been observed in response to salt treatment (Botella *et al.,* 1996). Sorghum roots exposed to osmotic stress for 1 hr exhibited increased CDPK activity *in vitro* (Pestenacz and Erdei, 1996).

In *Arabidopsis,* the expression of a gene encoding a phosphatidylinositol-specific phospholipase C is induced by several environmental stresses, including dehydration and salinity (Hirayama *et al.,* 1995), suggesting that phospholipase C is used in the signal transduction pathways involved in the responses to these stimuli. Phospholipase C (PLC) is the plasma membrane enzyme that releases IP_3 from membrane phospholipid. IP_3 is known to mediate the release of Ca^{2+} from internal stores, and therefore it has been suggested that a stress-induced PLC gene be involved in the amplification of the stress signals during stress acclimation by increasing the magnitude of the stress-induced calcium signal (Hirayama *et al.,* 1995).

The *sos3* mutation has been described in *Arabidopsis* and renders plants sensitive to Na^+-induced growth inhibition (Liu and Zhu, 1997). This effect is specific to Na^+ and lithium ions and does not occur in response to other cations or osmotic stresses. The mutant phenotype can be suppressed by high levels of external Ca^{2+} (Liu and Zhu, 1997). The *sos3* gene encodes a protein that is very similar in sequence to the calcineurin B subunit of yeast and neuronal calcium sensors (Liu and Zhu, 1998), and it has been suggested that SOS3 may be a calcium sensor, responding to salt-induced increases in $[Ca^{2+}]_{cyt}$ and mediating plant responses to salinity (Liu and Zhu, 1998). Transgenic tobacco expressing a modified form of calcineurin with constitutive phosphatase activity have been shown to have increased salinity tolerance, implicating calcineurin in the response to salt (Pardo *et al.,* 1998).

1. Effect of Salinity and Drought Stress on $[Ca^{2+}]_{cyt}$

Measurements of the effect of salinity on membrane-associated Ca^{2+} preceded direct measurement of $[Ca^{2+}]_{cyt}$ in response to drought or salinity. The fluorescent probe chlortetracycline (CTC) was used to demonstrate the involvement of Ca^{2+} in the response to salinity. Experiments using CTC to measure membrane-bound Ca^{2+} showed that NaCl reduced fluorescence, indicating that salinity displaced Ca^{2+} from cellular membranes of cotton root cells (Cramer *et al.,* 1985). This response to Na^+ was not seen with other cations nor in response to mannitol and therefore was assumed to be specific to Na^+ ions (Cramer *et al.,* 1985). Increasing the external concentration of Ca^{2+} to 10 m*M* reduced the effect of Na^+ (Cramer *et al.,* 1985).

The quenching of CTC fluorescence by NaCl occurred also on addition of EGTA (Cramer *et al.,* 1985). This indicated that the membrane-bound Ca^{2+} was accessible from the outside of the cell, and therefore the authors of this study concluded that Na^+ ions displaced Ca^{2+} from the plasma membrane (Cramer *et al.,* 1985). Work with cotton seedlings confirmed that $^{45}Ca^{2+}$ influx across the plasma membrane increased under salinity treatment (Cramer *et al.,* 1987); however, further evidence emerged to support the suggestion that Ca^{2+} from internal stores was mobilized during salinity treatment as well as external Ca^{2+} (see below). In contrast with earlier claims that Na^+ competes directly with Ca^{2+} for binding sites at the plasma membrane, data from a study using $^{22}Na^+$ in maize roots indicated that this was not the case (Zidan *et al.,* 1991). Ca^{2+} reduced the influx of Na^+, but the kinetics of this effect indicated that a competitive interaction between the two ionic species was not involved (Zidan *et al.,* 1991). Alternative explanations for the effect of Ca^{2+} on Na^+ influx include the suggestion that Ca^{2+} may increase the selectivity of plasma membrane K^+ channels, reducing Na^+ influx via this route, or that Ca^{2+} may have a general stabilizing effect on membrane structure (Zidan *et al.,* 1991).

There is significant evidence that Ca^{2+} release from intracellular stores plays a role in the responses both to salinity and osmotic stress or drought responses. The results from a study using CTC in maize root protoplasts indicated that the main effect of salinity was actually on intracellular calcium with a lesser effect on plasma membrane-bound Ca^{2+} (Lynch and Läuchli, 1988). Data showing that salinity could displace membrane-bound Ca^{2+} from protoplasts confirmed that the effect of NaCl was not dependent on apoplastic Ca^{2+} (Lynch *et al.,* 1987). The effect of sodium chloride treatment on CTC fluorescence was reduced by treatment with lithium, an inhibitor of phospholipid cycling, and restored by addition of inositol. These data strongly suggested that intracellular calcium release could be mediated by IP_3 (Lynch and Läuchli, 1988). In a subsequent study, the Ca^{2+}-sensitive fluorescent dye indo-1 was used to directly demonstrate that salinity causes an immediate increase in intracellular Ca^{2+} concentration in maize root protoplasts (Lynch *et al.,* 1989), and this elevation of $[Ca^{2+}]_{cyt}$ was reduced by pretreatment with lithium. Results obtained with aequorin-expressing *Arabidopsis* support these data (see below). Together, these data suggested a role for calcium signaling, probably involving intracellular and extracellular sources of Ca^{2+}, and a role for inositol phosphates in the response to salinity.

In *Chara,* Ca^{2+} influx increased in response to salinity, measured with $^{45}Ca^{2+}$ (Reid *et al.,* 1993). This effect appeared to be due to increased turgor and was not Na^+ specific (Reid *et al.,* 1993). Using radiolabeled Ca^{2+}, it was shown that salt stress caused an increase in external calcium influx in

the unicellular alga *Duniella salina* and that the magnitude of this influx depended on the degree of salt stress (Ko and Lee, 1995).

Further reports of $[Ca^{2+}]_{cyt}$ elevations in response to salinity include the demonstration of salt-induced $[Ca^{2+}]_{cyt}$ in barley root protoplasts and in wheat aleurone cells, loaded with the Ca^{2+}-sensitive fluorescent dyes indo-1 and fluo-3, respectively (Bittisnich *et al.,* 1989; Bush, 1996). Data obtained from experiments with *Arabidopsis* roots loaded with the ratiometric Ca^{2+}-sensitive dye, fura-2, indicated that in this tissue, NaCl treatment caused a decrease in cytosolic Ca^{2+} levels (Cramer and Jones, 1996). These authors attributed this contrasting result to the fact that intact roots were used rather than isolated protoplasts as in previous salinity studies (Bittisnich *et al.,* 1989; Lynch *et al.,* 1989). However, experiments using intact *Arabidopsis* seedlings (see below) showed increases in $[Ca^{2+}]_{cyt}$ (Knight *et al.,* 1997). The problems of variations in response from cell to cell which were encountered in these experiments may also contribute to these apparently contrasting results (Cramer and Jones, 1996). It would seem most likely that these different observations occurred due to the timing of the measurements. The elevation of $[Ca^{2+}]_{cyt}$ in response to salinity in the aequorin study occurred within seconds of stimulation (Knight *et al.,* 1997) whereas the decrease in $[Ca^{2+}]_{cyt}$ measured using fura-2 was reported to occur within 5 min (Cramer and Jones, 1996). It is possible therefore, that a decrease in the resting level of $[Ca^{2+}]_{cyt}$ occurs minutes after the initial transient increase in $[Ca^{2+}]_{cyt}$.

Elevations in $[Ca^{2+}]_{cyt}$ in response to NaCl were measured in whole intact tobacco seedlings using the recombinant aequorin method (Knight *et al.,* 1991). Further investigations with whole *Arabidopsis* seedlings revealed that under the conditions used very similar Ca^{2+} transients resulted from salinity treatment or treatment with an osmotically equivalent dose of mannitol at two different concentrations of salt or mannitol (Knight *et al.,* 1997). Measurements made using plants expressing aequorin targeted to the tonoplast membrane (Knight *et al.,* 1996) indicated that the responses to salt or mannitol treatment involved a significant contribution of Ca^{2+} released from the vacuole (Knight *et al.,* 1997). The effect of inhibitors of phosphoinositide cycling, lithium, U-73122, and neomycin, showed that this vacuolar released Ca^{2+} was likely to be triggered by IP_3-mediated release (Knight *et al.,* 1997), in agreement with earlier studies which suggested intracellular release of Ca^{2+} (Bittisnich *et al.,* 1989; Lynch and Läuchli, 1988; Lynch *et al.,* 1989).

2. Evidence for the Role of Ca^{2+} in Mediating Plant Responses to Drought and Salinity

The halotolerant unicellular alga *Duniella salina* accumulates glycerol in response to salt stress (Ko and Lee, 1995). Measurement of the amount of

glycerol accumulation by the alga in media containing different amounts of Ca^{2+} showed that there was a strong dependence of this response on Ca^{2+}. In the absence of Ca^{2+} the accumulation of glycerol was significantly reduced (Ko and Lee, 1995). The calmodulin antagonists TFP and W7 also caused a reduction in hyperosmotic stress-induced glycerol accumulation, suggesting the possible role of calmodulin or CDPKs in the response to salt stress (Ko and Lee, 1995).

The role of Ca^{2+} has been implicated in a number of higher plant studies also. The effects of supplemental Ca^{2+} supply on the response of sorghum seedlings to sodium chloride salinity treatment was studied (Colmer *et al.,* 1994). Two days of exposure to 150 m*M* NaCl resulted in a 50-fold increase in proline levels in root tips supplied with 5 m*M* Ca^{2+} but only a 4-fold increase in those supplied with 0.5 m*M* Ca^{2+}. It was subsequently shown that Ca^{2+} is involved in the signaling events leading to proline biosynthesis (Knight *et al.,* 1997).

Direct demonstration of the role of Ca^{2+} in salt- and drought-induced gene expression have been made in a number of systems. *RAB* (responsive to ABA) gene expression was measured in rice cell suspensions, in response to mannitol treatment (Leonardi *et al.,* 1995). Treatment with the calcium channel blocker verapamil reduced the levels of dehydration- and mannitol-induced *RAB* gene expression (Leonardi *et al.,* 1995).

Two membrane homologues of major intrinsic proteins (MIPs) have been identified in spinach leaf (Johansson *et al.,* 1996). The MIP group of proteins are channel forming proteins. The two homologues, PM28A and PM28B, were identified as putative aquaporins (water channels) and constitute the major phosphorylated polypeptide of spinach leaf plasma membrane. The levels of phosphorylation of the polypeptides *in vivo* changed in response to osmotic stress. The phosphorylation of PM28A *in vitro* was shown to be regulated by Ca^{2+}. As the activity of another MIP, α-TIP, has previously been shown to be regulated by phosphorylation (Maurel *et al.,* 1995), it has been suggested that Ca^{2+} acts to modify the phosphorylation status of these putative aquaporins and thus regulate their activity and resultant water uptake in response to osmotic stress (Johansson *et al.,* 1996). The involvement of Ca^{2+} in the phosphorylation and possible regulation of opening and closure of the channel highlighted its potential role as a transducer of water stress signals (Johansson *et al.,* 1996).

In whole intact *Arabidopsis,* expression of *AtP5CS1* (a salt- and drought-inducible gene (Savouré *et al.,* 1995; Yoshiba *et al.,* 1995) was measured in response to mannitol and NaCl (Knight *et al.,* 1997). Measurement of Ca^{2+} transients in response to equiosmolar concentrations of NaCl and mannitol had shown them to be very similar (see above) (Knight *et al.,* 1997). The *AtP5CS1* gene encodes an enzyme, Δ^1-pyrroline-5-carboxylate synthetase, the key regulating enzyme in the proline biosynthesis pathway (Delauney

and Verma, 1993). The equiosmolar mannitol and NaCl treatments both induced expression of *AtP5CS1,* and in both cases the amount of gene expression was reduced by treatment with EGTA or lanthanum. *AtP5CS1* expression was also reduced by the Ca^{2+} channel blocker verapamil and by gadolinium, the latter of which blocks stretch-activated calcium channels (Knight *et al.,* 1997). These data indicated that Ca^{2+} influx was required for full expression of the gene (Knight *et al.,* 1997). However, the levels of *AtP5CS1* expression were much lower in response to salinity than to drought, indicating that factors other than the size of the $[Ca^{2+}]_{cyt}$ elevation were contributing to the two pathways. One of the possible reasons for this difference is that, unlike NaCl, mannitol cannot be taken up by plant cells therefore cannot contribute to osmotic adjustment; thus, although its immediate effect on Ca^{2+} signaling may be similar, its effect in the longer term is more severe (Serrano, 1996). It is also possible that components in the signal transduction pathways include different elements in the case of drought or salt. ABA is known to be involved in the mediation of drought responses and may possible be one of the signaling components that distinguishes the pathways leading to salt- or drought-induced *AtP5CS1* gene expression. Differences in the transduction pathways involved in dehydration and osmotic stress have been demonstrated in rice cell suspensions (Leonardi *et al.,* 1995), and salt stress has also been shown to cause different effects on gene expression to those seen in response to osmotic stress (Tsiantis *et al.,* 1996). When the effects of water stress or ABA and osmotic stress (application of sorbitol) were compared in detached rice leaves, the stresses were found to act differentially on the levels of putrescine and proline (Chen *et al.,* 1994; Chen and Kao, 1993), indicating that these pathways may have both common and differing elements. It is likely, therefore, that the difference between the effects of drought, salinity, and water stress may involve other factors in addition to Ca^{2+}.

B. Hypoosmotic Shock

Osmotic stress is also experienced by plants in the form of hypotonic stress, that is, a reduction in osmotic potential. This is particularly true in the case of intertidal organisms, which experience constant fluctuations in the osmotic potential of their surroundings. The salt-tolerant alga *Chara longifolia* regulates its cell turgor in response to hypotonic stress resulting from a decrease in the osmotic pressure of the surrounding medium (Bisson *et al.,* 1995). In the first 5 min of hypotonic stress, the membrane depolarizes in a voltage-dependent fashion and the electrical conductance of the membrane increases transiently. When Ca^{2+} in the medium was lowered, the conductance did not alter, suggesting Ca^{2+} was necessary to mediate this

response. When Ca^{2+} influx was measured using $^{45}Ca^{2+}$, increased influx was noted during turgor regulation, occurring during the first 30–90 sec of hypotonic stress (Bisson *et al.,* 1995).

Transcellular osmosis is the term used to describe the condition which arises when a characean cell is partitioned into two pools of differing osmotic pressures (Tazawa *et al.,* 1994). When transcellular osmosis was imposed on cells of the alga *Nitella flexilis* injected with aequorin, an increase in luminescence was observed, indicating a transient elevation of $[Ca^{2+}]_{cyt}$ (Tazawa *et al.,* 1994). In similar experiments with *N. flexilis,* cytoplasmic hydration was effected by perfusing the vacuole with a hypotonic solution (Tazawa *et al.,* 1995). Increases in $[Ca^{2+}]_{cyt}$ were measured using aequorin luminescence and were also inferred by observation of cytoplasmic streaming, a process known to be inhibited by elevated Ca^{2+} concentrations (Kikuyama *et al.,* 1993). In both studies, hypoosmotic shock-induced increases in aequorin luminescence and cessation of cytoplasmic streaming were not inhibited by pretreatment of cells in EGTA, suggesting the involvement of internal Ca^{2+} stores (Tazawa *et al.,* 1994, 1995). Similar results have been obtained with other characean algae (Shimada *et al.,* 1996), and hypoosmotic treatment has also been demonstrated to increase the influx of $^{45}Ca^{2+}$ in the green alga *Duniella salina* (Ko and Lee, 1995). Hypotonic treatment induces turgor regulation in the alga *Lamprothamium* also, and induces a transient rise in $[Ca^{2+}]_{cyt}$ (Osaki and Iwasaki, 1991). The lag time for inhibition of cytoplasmic streaming after hypotonic treatment was prolonged in cells treated with EGTA, and the regulation of turgor pressure itself was partially inhibited (Osaki and Iwasaki, 1991). These data, as in the case of other data from algae, suggested that Ca^{2+} is involved in the control of turgor regulation (Osaki and Iwasaki, 1991).

Hypotonic shock of the marine alga *Fucus* zygote elicited an increase in $[Ca^{2+}]_{cyt}$ in the extreme rhizoid apex, which subsequently spread to the subapical regions as the rhizoid swelled in response to the change in osmoticum (Taylor *et al.,* 1996). It was demonstrated that Ca^{2+} influx occurs specifically in the apical rhizoid region during hypoosmotic shock, whereas in the subapical regions Ca^{2+} appeared to be released from internal Ca^{2+} stores (Taylor *et al.,* 1997), as deduced from previous algal studies (see above). The $[Ca^{2+}]_{cyt}$ transient was blocked by the stretch-activated channel blocker Gd^{3+} as was hypoosmotic shock-induced swelling (Taylor *et al.,* 1997). Microinjection of the Ca^{2+} buffer dibromo-BAPTA reduced the ability of zygotes to osmoregulate themselves, suggesting Ca^{2+} was required for this response (Taylor *et al.,* 1996). These data indicated that hypoosmotic stress activates both internal Ca^{2+} channels and mechanically sensitive plasma membrane Ca^{2+} channels and that the resultant $[Ca^{2+}]_{cyt}$ elevation is necessary for osmoregulation in the zygote.

In suspension culture cells of *Nicotiana tabacum* (tobacco) expressing recombinant aequorin, the effect of hypotonic shock was studied (Takahashi *et al.,* 1997), and the Ca^{2+} response was found to be composed of two separate phases. The first part of the response occurred immediately after dilution of the osmoticum and consisted of a small transient elevation; the second elevation was much larger and began about 30 sec after stimulation (Takahashi *et al.,* 1997). The phase 2 response required the continued presence of the hypotonic stimulus. The size of the response was directly related to the difference in osmolarity between the starting and end solutions. The size of the responses in phases 1 and 2 were dependent on the concentration of external calcium supplied in the medium (Takahashi *et al.,* 1997). The $[Ca^{2+}]_{cyt}$ elevation was not affected by the inhibitors of voltage-gated Ca^{2+} channels verapamil, nifedipine, and diltiazem at 5 μM, nor by bromophenacyl bromide (10 μM) or neomycin (100 μM), inhibitors of phosphoinositide metabolism and IP_3 synthesis, respectively (Takahashi *et al.,* 1997). The phase 2 response was inhibited by the protein kinase inhibitor K252a. These data suggested that phase 2 requires at least one phosphorylation event, although other kinase inhibitors tested did not have an effect. Gadolinium and lanthanum chloride were found to have markedly inhibitory effects on both phase 1 and phase 2 $[Ca^{2+}]_{cyt}$ elevations, suggesting a requirement for Ca^{2+} influx across the plasma membrane (Takahashi *et al.,* 1997).

IV. Oxidative Stress

A. Active Oxygen Species in Plants

Oxidative stress occurs in the response of plants to a wide variety of stresses, both environmental and biological. Recognition of avirulent pathogens causes an oxidative burst that leads to the mobilization of defense responses (Baker and Orlandi, 1995) including cross-linking of cell wall components (Keller *et al.,* 1998). The abiotic stresses leading to oxidative burst production include mechanical stimulation, high and low temperatures (particularly in combination with high light intensities), drought, ozone pollution, UV-B radiation, and herbicide application (Inze and Van Montagu, 1995). During oxidative stress excess production of active oxygen species (AOS) occurs, including production of superoxides ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$) which react with and damage cellular macromolecules (Bowler *et al.,* 1992; Inze and Van Montagu, 1995). Superoxide radicals and H_2O_2 can react to produce additional AOS that also cause damage to cell components. H_2O_2 and $O_2^{\cdot-}$ inactivate some macromol-

ecules, but they are most harmful through their conversion to OH· radicals, which react with DNA, proteins, and lipids causing cellular damage (Inze and Van Montagu, 1995). As the highly reactive OH· radicals cannot be eliminated enzymatically, plants defend themselves from the ill effects of oxidative stress by preventing OH· radical formation by scavenging O_2^{-}· and H_2O_2 (Inze and Van Montagu, 1995). Superoxide dismutases (SODs), of which a number of different forms exist (Kliebenstein Daniel *et al.,* 1998), eliminate O_2^{-}· while catalases and peroxidases eliminate H_2O_2. Plants also use free radical scavengers such as carotenoids and glutathione (Price *et al.,* 1994).

Active oxygen species and H_2O_2 have themselves been postulated to be signaling intermediates in their own right (Inze and Van Montagu, 1995). In maize, chilling tolerance could be induced by exposure to low levels of H_2O_2, suggesting a possible signaling role for this molecule (Prasad *et al.,* 1994b).

B. Effects of Oxidative Stress on Ca^{2+} Homeostasis and Control of Downstream Events

1. Chemically Induced Oxidative Stress

The effect of the pollutant gas ozone on Ca^{2+} transport was studied using vesicles from pinto bean leaves (Castillo and Heath, 1990). Ozone caused an increase in Ca^{2+} transport to the cytoplasmic side of the vesicles, the magnitude of the influx being dependent on the kinetics of the ozone exposure (Castillo and Heath, 1990). In one treatment, the gas was applied as a square wave of exposure during which the ozone concentration was immediately raised, sustained for 4 hr, and then decreased immediately. The effects of this square wave of exposure were compared to those of a triangular wave, characterized by a slow rise in ozone concentration to reach a final peak, and then a slow decline (Castillo and Heath, 1990). Both treatments involved equivalent total doses of ozone. However, the triangular phase method of application caused a larger increase in Ca^{2+} transport and was also more injurious to leaves than the square phase (Castillo and Heath, 1990).

The effects of oxidative stress on guard cells of *Commelina communis* were investigated by application of H_2O_2 (which generates hydroxyl radicals directly) and methyl viologen (The herbicide paraquat, which generates hydroxyl radicals as a secondary AOS after the formation of superoxide radicals) (McAinsh *et al.,* 1996). Using fluorescence ratio photometry of the calcium-sensitive dye fura-2, the authors measured changes in guard cell $[Ca^{2+}]_{cyt}$ in response to these two chemicals. Both methyl viologen

(MV) and H_2O_2 inhibited stomatal opening and promoted stomatal closure. EGTA completely abolished the effect of both methyl viologen and H_2O_2 on the opening and closing processes, when these chemicals were applied at concentrations of $<10^{-5}$ *M* MV or H_2O_2 (McAinsh *et al.,* 1996). At relatively higher concentrations, EGTA had little or no effect on the oxidative stress-mediated alterations in opening and closure (McAinsh *et al.,* 1996). These data indicated that the effects of oxidative stress on guard cells were mediated by Ca^{2+}, except at high concentrations of MV or H_2O_2. Further data indicated that this lack of Ca^{2+} dependency in response to more severe doses of oxidative stress may be due to general damaging of membranes and resultant loss of Ca^{2+} homeostasis. Guard cells exposed to concentrations of 10^{-5} *M* MV or H_2O_2 responded with a rapid rise in $[Ca^{2+}]_{cyt}$. These $[Ca^{2+}]_{cyt}$ elevations were categorized in to three classes: small, medium, and large responses, large being greater than 500 n*M* above the resting level and being a sustained increase (McAinsh *et al.,* 1996). Increases in $[Ca^{2+}]_{cyt}$ in response to either MV or H_2O_2 were inhibited by EGTA, suggesting that calcium entering from outside the cell was necessary for the response. These data indicate that at the relatively lower concentrations used, MV and H_2O_2 both elicit $[Ca^{2+}]_{cyt}$ elevations in stomatal guard cells and that these transients control the processes limiting guard cell opening (McAinsh *et al.,* 1996).

In contrast to the increases in Ca^{2+} transport reported in the response to oxidative stress (above), BCC1, a putative mechanosensory Ca^{2+} channel located in the endoplasmic reticulum (ER) of *Bryonia dioica,* was completely inhibited when 1 m*M* H_2O_2 was added to the cytoplasmic side. These data may indicate that the observed increases in $[Ca^{2+}]_{cyt}$ are not a result of Ca^{2+} release from the ER (Klüesener *et al.,* 1997).

The effect of oxidative stress on Ca^{2+} signaling in whole plants was studied using tobacco seedlings expressing recombinant aequorin (Price *et al.,* 1994). A rapid transient elevation of $[Ca^{2+}]_{cyt}$ was elicited by treatment with 10 m*M* hydrogen peroxide. This elevation could be inhibited both by lanthanum and by ruthenium red, which inhibits release from internal Ca^{2+} stores, indicating that both intracellular and extracellular Ca^{2+} stores contribute to the Ca^{2+} elevation (Price *et al.,* 1994). The authors observed a refractory period during which further stimulation would not elicit a response. The recovery period had a duration of approximately 4 to 8 hr, with recovery beginning after 30 min (Price *et al.,* 1994). To investigate the mechanism of hydrogen peroxide perception, several inhibitors of the pathways surrounding AOS formation were used. Buthionine sulfoxime (BSO) is an inhibitor of glutathione (GSH) synthesis, and hydroxylurea (HU) and hydroxylamine (HA) inhibit ascorbate peroxidase, the first enzyme of the Halliwell–Asada pathway which degrades hydrogen peroxide. Both groups of chemicals should retard the rate of hydrogen peroxide

metabolism, allowing a longer period over which hydrogen peroxide can cause $[Ca^{2+}]_{cyt}$ elevations. However, the effect of BSO on the prooxidant/antioxidant ratio is the opposite of that caused by HA or HU. (The primary prooxidants referred to here are dehydroascorbic acid, quinones, and glutathione and the antioxidants are the equivalent reduced forms.) BSO (which should elevate the prooxidant/antioxidant ratio) caused an increase in the peak height of the hydrogen peroxide-induced Ca^{2+} response, whereas HU and HA (which would be predicted to lower the prooxidant/antioxidant ratio) caused the Ca^{2+} response kinetics to become slower, with a lower peak value and a slower decline (Price *et al.,* 1994). These results indicated that the H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevations occurred not in direct response to H_2O_2 itself but in response to the prooxidant/antioxidant ratio. The prooxidant/antioxidant ratio has been reported to have an important homeostatic role in Ca^{2+} in animal cells also (Price *et al.,* 1994). At 10 m*M,* H_2O_2 inhibited SOD enzyme activity, causing a significant reduction after 4 hr. This effect was inhibited by lanthanum, suggesting that the Ca^{2+} elevation was important in controlling SOD activity levels in response to oxidative stress (Price *et al.,* 1994).

Using recombinant aequorin-expressing plants, the effects of ozone on tobacco (H. Clayton *et al.,* unpublished results) and *Arabidopsis* have been investigated (Clayton *et al.,* 1999). In both species Ca^{2+} elevations have been shown to be elicited by ozone. In both species a biphasic Ca^{2+} response occurs consisting of a relatively short Ca^{2+} spike soon after the onset of ozone fumigation and a slower and more sustained subsequent elevation over a period of about 1 hr (Clayton *et al.,* 1999). The second of these two $[Ca^{2+}]_{cyt}$ elevations occurred only when the concentration of ozone applied exceeded a critical threshold concentration. Ozone treatment also induced expression of genes encoding glutathione synthase (*GST*), proteins which confer protection against oxidative damage. Use of lanthanum chloride and EGTA showed that expression of these genes was Ca^{2+} regulated and that the second of the two $[Ca^{2+}]_{cyt}$ peaks was responsible for control of expression (Clayton *et al.,* 1999).

2. Blue Light/Ultraviolet-Induced Oxidative Stress

Exposure of *Arabidopsis* to UV-B induces the *CHS* gene, which encodes the enzyme chalcone synthase. Expression induced by UV-B irradiation was found to be inhibited in *Arabidopsis* cell suspensions by application of the voltage-dependant Ca^{2+} channel blocker nifedipine, but not by the plasma membrane channel blocker lanthanum or by verapamil (Christie and Jenkins, 1996). These data indicated that Ca^{2+} may be involved in the control of *CHS* expression in response to this stress, but that internal stores of Ca^{2+} were more likely to play a role than external stores. This

interpretation was strengthened by the finding that UV-B light-induced expression was inhibited by the putative intracellular Ca^{2+} channel blocker, ruthenium red, although its effect may not be confined to intracellular channels. Cells incubated in the Ca^{2+} ionophore A23187 did not show increased levels of *CHS* gene expression in the absence of UV treatment, indicating that although Ca^{2+} appeared to be involved in the response, an elevation of Ca^{2+} alone was not sufficient to induce expression (Christie and Jenkins, 1996). The effect of the inhibitor W7 was to reduce levels of UV-B-induced *CHS* expression, indicating that calmodulin or a CDPK was involved in the UV-B pathway. The protein kinase inhibitors K252a and staurosporine completely inhibited *CHS* expression in response to UV-B. The effect of the protein phosphatase inhibitor okadaic acid, which inhibits protein phosphatases 1 and 2A, was also to inhibit *CHS* expression in response to UV-B. Therefore, a role for both protein phosphatase and kinase activity was implicated in the pathway leading to UV-B induction of *CHS* gene expression (Christie and Jenkins, 1996).

Further elucidation of the role of Ca^{2+} in regulation of responses to UV-A and UV-B was performed using expression of the calcium-regulated gene *TCH3* as a marker for elevations of $[Ca^{2+}]_{cyt}$ (Long and Jenkins, 1998). Ca^{2+} ionophore treatment caused an increase in *TCH3* expression but this induction was strongly reduced if cells were treated with UV-A/blue or UV-B light, which suggested that these two light qualities may cause efflux of Ca^{2+} from the cytosol (Long and Jenkins, 1998). The Ca^{2+}-ATPase inhibitor erythrosine B strongly inhibited the expression of *CHS* in response to UV-A/blue light but had no effect on the UV-B-induced expression of this gene (Long and Jenkins, 1998). These data suggested that erythrosine B inhibits the activity of a Ca^{2+}-ATPase specifically in UV-A/blue light, in which case $[Ca^{2+}]_{cyt}$ would be expected to rise. Increased levels of *TCH3* expression occurring in erythrosine B-treated cells illuminated with UV-A/blue light indicated that this was the case. A lack of *TCH3* expression in cells treated with the inhibitor and UV-B confirmed that this effect was specific to UV-A/blue light (Long and Jenkins, 1998). The calmodulin inhibitor W7 overcame the inhibitory effect of UV-B on *TCH3* expression, suggesting a calmodulin-regulated Ca^{2+}-ATPase was involved. As a whole, the results of these studies indicate that different types of Ca^{2+}-ATPase are involved with UV-A/blue light and UV-B signaling pathways (Long and Jenkins, 1998).

3. Evidence of Ca^{2+} Regulation of Oxidative Burst Components

Oxidative stress, in the form of chilling, mechanical stimulation, light, or gaseous treatments, causes an increase in $[Ca^{2+}]_{cyt}$, and this may potentially

control a variety of events including the induction of protective responses and initiation of the oxidative burst itself. The regulation of components controlling the oxidative burst has been studied and a possible role for Ca^{2+} has emerged. In one study, the roles of calmodulin and NAD kinase in the generation of AOS were investigated (Harding *et al.,* 1997). This study employed transgenic tobacco cell cultures expressing a dominant-acting mutant calmodulin VU-3, which cannot be trimethylated posttranslationally. The result of this modification is that most of the calmodulin functions remain normal except that the VU-3 calmodulin hyperactivates calmodulin-dependent NAD kinase (Harding *et al.,* 1997). The cells expressing the modified calmodulin exhibited a stronger than normal oxidative burst in response to a range of biological elicitors as well as to mechanical or osmotic stimulation (Harding *et al.,* 1997). The oxidative burst in the VU-3 mutant also occurred more rapidly. Increases in NADPH levels were greater in VU-3 than in wild-type cells and corresponded with the timing and magnitude of the oxidative burst. These results indicated that calmodulin is a target of oxidative stress-induced Ca^{2+} elevations and suggested that a plant NAD kinase may be a downstream target in this pathway, facilitating AOS production via an NADPH-dependent pathway (Harding *et al.,* 1997).

Further evidence for a role for Ca^{2+} in generating the oxidative burst has arisen from the discovery of a plant gene, *RBOHA,* which encodes a putative intrinsic plasma membrane NADPH oxidase that is similar to the neutrophil NADPH oxidase $GP91^{PHOX}$ and which contains calcium biding sites (Keller *et al.,* 1998). The authors proposed that RbohA is a Ca^{2+}-regulated plasma membrane enzyme involved in the rapid generation of $O_2^{\cdot-}$ and H_2O_2 (Keller *et al.,* 1998). When an oxidative burst was artificially induced in *Egeria densa* by the addition of the sulfhydryl blockers *N*-ethylmaleimide (NEM) or Ag^+, there was a concomitant production of H_2O_2 (Marrè *et al.,* 1998). The presence of Ca^{2+} in the medium during stimulation was strictly required for these effects to occur. The authors of this work demonstrated that NEM stimulation caused an influx of Ca^{2+} which was blocked by La^{3+} (Marrè *et al.,* 1998). La^{3+} treatment also inhibited the H_2O_2-inducible increase in electrolyte leakage. These data provided further evidence that oxidative stress-induced Ca^{2+} fluxes regulate the activity of an NADPH oxidase (Marrè *et al.,* 1998).

As well as a role for Ca^{2+} in generating an oxidative burst, there is evidence for a role in activating scavenging mechanisms. The barley grain peroxidase BP1 reacts with H_2O_2 with reaction kinetics involving a slow and a fast reaction occuring simultaneously, resulting in biphasic kinetics. The reaction is monophasic in the absence of Ca^{2+}, but the addition of calcium causes BP1 to catalyze the rapid reaction too, resulting in biphasic kinetics (Rasmussen *et al.,* 1998). The BP1 enzyme has Ca^{2+} binding sites

and, on binding, becomes the fast acting form by undergoing Ca^{2+}-induced conformational changes (Rasmussen *et al.,* 1998).

V. Anoxia

Anoxia, oxygen deprivation, is the primary stress factor affecting plants in flooded soils (Subbaiah *et al.,* 1994a). When plants sense a reduction in oxygen concentration, expression of specific genes is induced during what appears to be an adaptive response to allow survival under these conditions (Subbaiah *et al.,* 1994a). A number of specific proteins are expressed during anoxia, including the enzyme alcohol dehydrogenase (ADH) (Andrews *et al.,* 1993; Sachs, 1991). During anaerobiosis, acidification of the cytoplasm occurs, and there is good evidence that this change in pH triggers some of the metabolic responses to anoxia (Ratcliffe, 1997). The activity of several enzymes involved in the response to anoxia has been reported as being controlled by pH, including pyruvate decarboxylase and glutamate decarboxylase (GAD), the enzyme which controls the production of GABA (Cholewa *et al.,* 1997; Ratcliffe, 1997). However, although pH changes had been thought to constitute the key controlling mechanism operating during responses to anoxia, it now seems more likely that the primary level of control is via Ca^{2+} (Ratcliffe, 1997). GAD, for instance, has also been shown to be activated by Ca^{2+} (Snedden *et al.,* 1995). As Ca^{2+} is implicated in a growing number of stress responses, so has the likelihood of a role for Ca^{2+} in anoxia signaling increased. It has now been demonstrated that anoxia elevates $[Ca^{2+}]_{cyt}$ (as described below) as well as $[H^+]_{cyt}$, and therefore it seems probable that Ca^{2+} acts as a second messenger during anoxia stress. A complex relationship exists between Ca^{2+} and pH (Irving *et al.,* 1992; Felle, 1988; Kinoshita *et al.,* 1995), making it possible that Ca^{2+} and pH have interrelated roles in control of the responses to anoxic stress.

An anoxia-induced increase in uptake of $^{45}Ca^{2+}$ was demonstrated in maize roots (Subbaiah *et al.,* 1994a). This increase was reduced by treatment with the Ca^{2+} channel blocker ruthenium red (RR), and a similar but milder effect was seen with the Ca^{2+} channel blockers nifedipine and verapamil (Subbaiah *et al.,* 1994a). Under anoxia, the glycolytic pathway has a key role for energy production. Continued oxidation of carbohydrate in anoxia depends on reoxidation of the NADH produced by glycolysis by conversion of pyruvate to lactate (in the short term) or to ethanol (in the longer-term) (Ratcliffe, 1997). The ethanol accumulated is broken down by ADH, the activity of which is induced severalfold in maize seedlings after flooding, and this response is essential for survival of anoxia (Schwartz, 1969). Therefore, measurement of ADH activity provides a useful biochemical marker for

the adaptive response to anoxia. When maize seedlings were submerged in water, ADH activity was induced, but this response was affected by disrupting Ca^{2+} homeostasis with either RR red or nifedipine. RR, but not nifedipine, also prevented recovery of seedlings from anoxia (Subbaiah *et al.,* 1994a). Survival of anoxia by RR-treated plants was improved when RR treatment was accompanied by the addition of Ca^{2+}, suggesting that Ca^{2+} mobilization was necessary for the induction of survival responses (Subbaiah *et al.,* 1994a). When levels of *ADH* gene expression were measured, RR was found to inhibit the levels of expression measured in response to anoxia (Subbaiah *et al.,* 1994a). The effect of RR on *ADH* gene expression or ADH activity levels was reversed if Ca^{2+} was supplied with the RR, suggesting that RR was acting by blocking specific Ca^{2+} fluxes. Treatment with the Ca^{2+} chelators EGTA or BAPTA did not reduce anoxia-induced *ADH* gene expression in contrast with the effect of the inhibitor RR, which is thought mainly to affect Ca^{2+} release from intracellular stores (Subbaiah *et al.,* 1994a). Together these data suggested that Ca^{2+} was important in the early events leading to the response of plants to anoxia, and that internal Ca^{2+} release appears to be important, as well as a possible influx of Ca^{2+}.

Alterations in $[Ca^{2+}]_{cyt}$ in response to anoxia have been measured directly in maize suspension-cultured cells loaded with the Ca^{2+}-sensitive fluorescent dye fluo-3. An immediate slow steady rise in $[Ca^{2+}]_{cyt}$ was observed in response to anoxia, and this elevation ceased and levels reduced when normoxia was restored; ''hot-spots'' were noted in the cytoplasm around the nucleus and subsequently spread throughout the cell. Incorporation of EGTA into the perfusion medium did not prevent the anoxic Ca^{2+} rise, suggesting that influx of external Ca^{2+} was not required for the anoxia-induced $[Ca^{2+}]_{cyt}$ elevation (Subbaiah *et al.,* 1994b). In contrast, RR did block the anoxia-induced $[Ca^{2+}]_{cyt}$ elevation, indicating that internal stores of Ca^{2+} were contributing to the anoxic $[Ca^{2+}]_{cyt}$ elevation. It was suggested, however, that influx of extracellular Ca^{2+} could be involved in restoring resting levels of Ca^{2+} in the cytosol after a depletion of internal Ca^{2+} stores by anoxia. More recently, work by these authors using suspension-cultured maize cells has shown that mitochondria release Ca^{2+} during anoxia and take Ca^{2+} up again on reoxygenation (Subbaiah *et al.,* 1998). Using a Ca^{2+}-sensitive fluorescent dye, it was demonstrated that in response to anoxia mitochondrial Ca^{2+} levels dropped, whereas $[Ca^{2+}]_{cyt}$ in the areas of cytoplasm around the mitochondria rose (Subbaiah *et al.,* 1998).

Measurement of $[Ca^{2+}]_{cyt}$ in whole intact *Arabidopsis* seedlings using the aequorin method confirmed findings from single cell studies and revealed more about the anoxia-induced $[Ca^{2+}]_{cyt}$ elevation (Sedbrook *et al.,* 1996). A typical whole seedling response consisted of a transient peak of aequorin luminescence beginning approximately 50 sec after the onset of anoxia and

lasting less than 10 min (referred to as peak I), followed by an immediate reduction to near baseline levels. This transient peak was followed by a second, slower increase in luminescence that reached a peak within 0.5–2 hr after initiation of anoxia (peak II). This peak declined slowly over 1–2 hr. Return to normoxia elicited a third peak (peak III) (Sedbrook *et al.,* 1996). The response of the different plant organs to anoxia was measured, revealing that the Ca^{2+} response observed in the whole seedling was attributable to the shoots and cotyledons of the plants; the roots and hypocotyl did not respond with peaks I or II. However, the roots and hypocotyl did show a small response on restoration of normoxia (Sedbrook *et al.,* 1996). The normoxia-induced peak was always preceded by a brief, transient reduction in luminescence levels.

Pretreatment with RR caused a lowering of the peak I luminescence and caused an earlier than normal peak II of greater amplitude than seen in nonpretreated plants. A smaller and later luminescence peak (peak III) was observed in response to normoxia in RR-pretreated plants. Gadolinium pretreatment was used to ascertain whether plasma membrane Ca^{2+} channels were involved in the Ca^{2+} response. The effect of this channel blocker was to reduce peak I (as did RR) but to cause a more rapid peak II to occur with a far greater magnitude. Gadolinium abolished the normoxia-induced peak III (Sedbrook *et al.,* 1996). Lanthanum also had a similar effect. EGTA caused some reduction of peak I but had a variable effect on peak II. EGTA too abolished the normoxia-induced peak. The effect of EGTA could be counteracted by coapplication of external Ca^{2+}, suggesting that influx of Ca^{2+} was involved in these parts of the response. The effects of these inhibitors on the $[Ca^{2+}]_{cyt}$ elevations suggested that both internal and external stores may contribute to peak I of the response, and that the response to restoration of normoxia may rely mainly on external stores (Sedbrook *et al.,* 1996).

The differences between the results gained from the single cell maize $[Ca^{2+}]_{cyt}$ measurements and the whole seedling *Arabidopsis* measurements may be due to differences in either the media constitution or the cell types; both of these factors were found to influence the response obtained from *Arabidopsis* measurements (Sedbrook *et al.,* 1996). They could, alternatively, be due to a masking of peak I in the cultured cells by a particularly early peak II. In other words, the single peak response seen in the cultured cells may represent a combination of the equivalents of peaks I and II of the *Arabidopsis* response. Anoxia-induction of *ADH* gene expression in *Arabidopsis* was inhibited by either gadolinium or RR, indicating involvement of Ca^{2+}. However, overall, luminescence changes did not correlate strictly with *ADH* expression: expression was seen in the roots where no Ca^{2+} signal was seen, and the inhibitors gadolinium and RR both increased peak II but reduced the levels of *ADH* expression. Therefore Ca^{2+} signaling

may not mediate *ADH* gene expression, although the data do not preclude a role for Ca^{2+} in facilitating other adaptive responses to anoxia.

Little has been described in the literature regarding the role of Ca^{2+} and Ca^{2+}-dependent signaling components in other anoxic responses. It has been reported that a Ca^{2+}-dependent protein kinase is activated during anoxia (Subbaiah and Sachs, 1993), and this may play a role in anoxia-induced activation of gene expression. Two genes isolated from rice coleoptiles, *OSCPK2* and *OSCPK11,* encode for putative CDPK proteins (Brevario *et al.,* 1995). Interestingly, *OSCPK2* mRNA levels in coleoptiles *decrease* after treatment with anoxia, although levels of *OSCPK11* are unaffected. The authors suggested that this downregulation of expression could be due to a lack of available substrate for the CDPK enzyme or alternatively may be part of an adaptive response to the anoxic stimulus. Whichever is the case, the fact that anoxia is regulating levels of a Ca^{2+} signal transduction protein is likely to be significant (Brevario *et al.,* 1995).

In rice seedlings subjected to anaerobic conditions for 2 min, cAMP levels in the shoot were strongly reduced (Reggiani, 1997). The concentration of cGMP increased 2.5-fold in 2 min (Reggiani, 1997). Similar anaerobiosis-induced transients were seen in the root. In mammalian cells an increase in cGMP levels is recognized as a signal in response to anoxia (Deprè and Hue, 1994). There is evidence that cAMP can directly stimulate Ca^{2+} channel activity without phosphorylation (Kurosaki and Nishi, 1993) and that $[Ca^{2+}]_{cyt}$ is elevated by addition of cAMP or cGMP (Volotovski *et al.,* 1998). It is therefore possible that cyclic nucleotides are effecting anoxic responses via modulation of anoxic Ca^{2+} signals.

VI. Heat Stress

Heat stress of plants has been known for some time to cause changes in transcription of mRNAs encoding heat shock proteins (HSPs) and to affect their selective translation. These changes in HSP expression result in increased thermotolerance (Howarth and Ougham, 1993). Thermotolerance can be induced in plants by pretreating them with a lower heating temperature than the one to be encountered at a later point. Induced thermotolerance can be initiated in maize seedlings by a pretreatment with temperatures ranging from 38° to 44°C, and it increased subsequent survival at 50°C (Gong *et al.,* 1997a). Presoaking of maize seeds in calcium chloride before germination markedly enhanced thermotolerance in the resulting seedlings (Gong *et al.,* 1997a), suggesting that Ca^{2+} may be required for the induction of thermotolerance. Presoaking the seeds in EGTA, lanthanum, or verapamil all reduced levels of acquired thermotolerance, as measured by subse-

quent treatment at 50°C. Calmodulin levels in seedlings increased during heat stress, suggesting that this too was involved in the acquisition of thermotolerance. Subsequent survival of seedlings was significantly reduced by addition of the calmodulin antagonists CPZ and W7. Similarly, levels of induced thermotolerance were significantly reduced by incubating seedlings in EGTA before the heat pretreatment period, but survival was enhanced by addition of Ca^{2+} to the medium, suggesting that Ca^{2+} is necessary for induced thermotolerance (Gong *et al.,* 1998).

In *Brassica napus,* the Ca^{2+} chelator EGTA and the calcium ionophore A23187 altered the heat-induced accumulation of a number of specific proteins in excised hypocotyl sections, suggesting a regulating role for Ca^{2+} (Wu *et al.,* 1992). Measurement of antioxidant enzyme activities and lipid peroxidation showed that heat stress induced an oxidative stress in maize seedlings (Gong *et al.,* 1997b). External Ca^{2+} treatment caused the seedlings to maintain relatively higher activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) and resulted in lower levels of heat-induced lipid peroxidation than Ca^{2+}-deficient treatments; on the contrary, EGTA treatment led to more rapid loss of SOD, CAT, and APX activities and higher levels of lipid peroxidation in the seedlings under heat stress. In addition, concurrent Ca^{2+} and W7 treatment weakened the effects of Ca^{2+} treatment on SOD, CAT, and APX activities and caused more severe lipid peroxidation. These results suggested that external Ca^{2+} can enhance the intrinsic heat tolerance of maize seedlings (Gong *et al.,* 1997b).

More evidence that Ca^{2+} may be involved in the response of plants to heat includes the observation that heat shock strongly induces expression of the *TCH* genes in cultured *Arabidopsis* cells, in a manner dependent on extracellular Ca^{2+} (Braam, 1992a). The TCH genes themselves encode calmodulin-like proteins, which may have an effect on Ca^{2+} homeostasis, suggesting that Ca^{2+} is involved in responses to heat. Other HSPs have been identified as calmodulin-binding proteins also (Lu *et al.,* 1995). The increased expression of such possible Ca^{2+} signaling components in response to heat would suggest that Ca^{2+} signaling could be involved in at least part of the response to elevated temperatures.

Heat-induced elevations of $[Ca^{2+}]_{cyt}$ were inferred by measurement of significantly enhanced levels of Ca^{2+} uptake by suspension-cultured pear cells and protoplasts after exposure to temperatures of 38°C (Klein and Ferguson, 1987). Direct measurement of heat shock-induced Ca^{2+} elevations has been performed in pea mesophyll protoplasts using the fluorescent dye indo-1 (Biyaseheva *et al.,* 1993). In these experiments heat shock induced a 4-fold increase in $[Ca^{2+}]_{cyt}$. Further studies of heat shock-induced Ca^{2+} elevations has been achieved in tobacco using the recombinant aequorin technique (Gong *et al.,* 1998). Heat shock of tobacco seedlings expressing

recombinant aequorin revealed that after 5 to 35 min at elevated temperatures (39°, 43°, or 47°C) $[Ca^{2+}]_{cyt}$ was increased. The elevation of $[Ca^{2+}]_{cyt}$ was prolonged but transient. Brief treatments with hot water did not elevate $[Ca^{2+}]_{cyt}$ (Gong *et al.,* 1998; Knight *et al.,* 1991). Preincubation with Ca^{2+} caused the heat-induced $[Ca^{2+}]_{cyt}$ elevation to begin more quickly, and to reach a higher than normal peak level. In contrast, pretreatment with EGTA caused a reduced and slower response (Gong *et al.,* 1998). The response was attenuated by repeated heat stimulation, although plants were able during this period to respond to other stresses normally, such as cold. The magnitude of the signal was inhibited by the channel blockers ruthenium red and lanthanum as well as by the phospholipase C inhibitor neomycin. These data suggested the possibility that both extracellular and intracellular (possibly IP_3-mediated) stores are involved in contributing to the $[Ca^{2+}]_{cyt}$ elevation.

VII. Mechanical Stress

Mechanical stimuli are experienced by plants at almost all times, through wind, rain, and being touched through encounters with neighboring plants or animals. Both external stimuli of this nature and changes in cell juxtapositioning that occur during growth cause tension and compression of cells (Trewavas and Knight, 1994), and constitute information regarding the internal and environmental conditions experienced by the plants' cells (Ding and Pickard, 1993b). Plants such as climbers and insectivorous plants show obvious and rapid responses to touch stimulation, but all plants exhibit the slower thigmomorphogenetic responses to mechanical stimulation (MS), including a reduction in height and increase in stem thickening (Erner and Jaffe, 1983; Trewavas and Knight, 1994). Such thigmomorphogenetic responses appear to be involved in hardening of the plant against further perturbations (Jaffe and Forbes, 1993). Perception of mechanical stimuli occurs not only in the aerial parts of the plant but also in roots, as shown by their ability to recognize mechanical stimulation and alter their direction of growth when encountering obstacles (Okada and Shimura, 1990).

A. The Involvement of Ca^{2+} in Responses to Mechanical Stimulation

There have been a number of reports in the literature suggesting that thigmomorphogenesis may involve changes in calcium homeostasis. Rubbing-induced growth inhibition of soybean stems was shown to be

decreased by treatment with EGTA or calmodulin-binding inhibitors, implicating Ca^{2+} in this particular thigmomorphogenic response (Jones and Mitchell, 1989), and both Ca^{2+}-chelating agents and lanthanum have been shown to have an inhibitory effect on the mechanical response of *Mimosa pudica* to touch (Campbell and Thomson, 1977). Thigmotropism of *Zea mays* roots was inhibited by approximately 65% when pretreated with gadolinium, an inhibitor of stretch-activated Ca^{2+} channels (Millet and Pickard, 1988). In the flagellate green alga *Spermatozopsis,* mechanical stimulation can cause a change from forward to rapid backward swimming. Depletion of Ca^{2+} from the medium led to a significant reduction in this response, indicating that Ca^{2+} was required for transduction of the mechanostimulatory signal (Kreimer and Witman, 1994). Touch-induced coiling in whole plants of *Bryonia dioica* was significantly reduced by erythrosine B, an inhibitor of Ca^{2+}-ATPases which also reduces activity of the BCC1 ER Ca^{2+} channel (Liss *et al.,* 1998).

Data of this kind have indicated that Ca^{2+} is necessary for these thigmotropic responses; however, some evidence is to the contrary, suggesting the requirement for Ca^{2+} is not straightforward. The stamens of *Berberis vulgaris* are a popular model for studying thigmonasty, as mechanical stimulation of the sensitive internal lower part of the filament of the stamen causes a rapid stamen movement resulting in the striking of the anther against the pistil of the flower (Lechowski and Bialczyk, 1992). When excised flowers were placed in solutions of differing concentrations of $CaCl_2$, the mechanical response was lost when the Ca^{2+} concentration reached 100 m*M,* indicating an inhibitory effect of extracellular Ca^{2+} (Lechowski and Bialczyk, 1992). The cessation of bending caused by Ca^{2+} externally applied could be reversed by application of a calcium-chelating agent such as EGTA. Such data suggest that under the conditions used, the addition of Ca^{2+} is inhibitory to this particular thigmotropic response; however, this may be due to too high a concentration of Ca^{2+} being applied or possibly to the intracellular location of the resultant rise in intracellular Ca^{2+} concentration.

As well as the requirement for Ca^{2+} for some thigmomorphogenetic responses, other evidence of a role for Ca^{2+} in responses to MS exists. Ca^{2+} is strongly implicated in such responses by reports of mechanical stimulation-induced changes in the expression of Ca^{2+} signaling components. In particular, expression of genes encoding calmodulin or calmodulin-related proteins are upregulated in response to mechanical stimulation in a number of species. A *Brassica napus* clone *BCM1* was expressed in response to mechanical stimulation (Oh *et al.,* 1996), and in *Bryonia* a mechanically induced cDNA *Bc329* was identified as encoding calmodulin (Galaud *et al.,* 1993). In potato *PCM1,* a calmodulin clone, was expressed in response to touch, although eight other calmodulin clones isolated did

not respond in this way (Takezawa *et al.,* 1995). In tomato also, calmodulin RNA accumulated in response to rubbing of the internodes (Depège *et al.,* 1997). The kinetics of touch-induced increases in expression of calmodulins can differ, suggesting that different isoforms are required at different times during the response to MS. In *Vigna radiata,* the calmodulin gene *MBCaM-1* mRNA levels responded dramatically with an increase in response to touch whereas *MBCaM-2* showed a small but steady increase (Botella and Arteca, 1994).

A number of calmodulins have been reported in *Arabidopsis* (Braam and Davis, 1990; Gawienowski *et al.,* 1993; Perera and Zielinski, 1992), of which several are MS induced. Three touch-induced calmodulin mRNAs (ACaM1, ACaM2, and ACaM3) were identified in *Arabidopsis* (Perera and Zielinski, 1992), and two other touch-induced genes, one encoding calmodulin and another encoding a calmodulin-related protein, were subsequently identified in *Arabidopsis* (Ito *et al.,* 1995). Four *TCH* (*touch*) genes have been identified in *Arabidopsis;* of these, *TCH1* encodes a calmodulin likely to be the gene for the same isoform as *AtCAL5* (Ito *et al.,* 1995), and *TCH2* and *TCH3* encode calmodulin-like proteins (Braam and Davis, 1990). The *TCH* genes are upregulated by touch and wind as well as by darkness (Sistrunk *et al.,* 1994). Expression of the *TCH* genes is strongly and very rapidly induced, reaching levels of 100-fold induction 10 to 30 min after touch stimulation (Braam and Davis, 1990) and returning to steady-state levels after 1 to 3 hr, suggesting a rapid turnover of these proteins (Braam *et al.,* 1996). *TCH3* encodes a protein similar to calmodulin that can bind up to 6 Ca^{2+} ions (Antosiewicz *et al.,* 1995). Expression of *TCH3* is also regulated developmentally, and it has been suggested that the TCH3 protein might function in a strengthening role during development (Antosiewicz *et al.,* 1995; Sistrunk *et al.,* 1994).

The possible functions of calcium-binding proteins, such as the TCH proteins, in signal transduction have been discussed and a number of possibilities are recognized. They may function to sequester Ca^{2+}, in a way similar to other calcium-binding proteins such as calmodulin, and thus aid the return to Ca^{2+} homeostasis after stimulus-induced Ca^{2+} elevations (Braam, 1992b). Alternatively, the proteins may act to regulate ion channel activities, a function that has been described for calmodulin in paramecium (Kung *et al.,* 1992), or may be involved in changing microtubule arrangements as described earlier. There is growing evidence implicating calmodulin as an important receptor that links changes in cellular calcium levels with cytoskeletal function (Zielinsky, 1998). Calmodulin is associated with cortical microtubule components of the plant cytoskeleton (Cyr, 1991). Elongation factor (EF) 1α is a microtubule-associated protein (MAP) target of calmodulin (Durso and Cyr, 1994). Calmodulin binds EF1α directly and thereby inhibits its ability to bundle microtubules *in vitro* (Durso and Cyr,

1994). It is likely, therefore, that calmodulin is a necessary component in the thigmomorphogenic responses to MS which involve changes in growth shape and form. From all these data, it appears that calmodulin is likely to play a role in the thigmomorphogenic response, possibly through its effect on the cytoskeleton and cell shape. This has been shown to be the case in other organisms; for instance, in *Paramecium,* calmodulin is involved in determining responses to touch stimulation (Kung *et al.,* 1992).

Other Ca^{2+}-related proteins that may feature in MS signaling include a CDPK in mung bean which is inducible by mechanical strain (Botella *et al.,* 1996) and annexins or annexin-like proteins (Thonat *et al.,* 1997). The cellular location of two annexin-like proteins (proteins that bind phospholipids in a Ca^{2+}-dependent manner) was studied in *Bryonia dioica* internodes, and it was discovered that MS caused a redistribution of one of these, p33, with the protein becoming localized to the plasma membrane after MS (Thonat *et al.,* 1997). These data suggest that other calcium-binding proteins may act in the MS signal transduction pathway, following an increase in $[Ca^{2+}]_{cyt}$.

B. The Mechanical Stimulation-Induced $[Ca^{2+}]_{cyt}$ Elevation

Using the recombinant aequorin method, it has been shown that $[Ca^{2+}]_{cyt}$ is elevated by touch stimulation (Knight *et al.,* 1991). Such elevations have been demonstrated in tobacco (Knight *et al.,* 1991, 1992, 1993), *Arabidopsis* (Knight *et al.,* 1995; A. J. Wright and M. R. Knight, in preparation), and *Physcomitrella* (Russell *et al.,* 1996). When increasing wind forces were applied to tobacco seedlings, a graded response was observed, with increases in the magnitude of the $[Ca^{2+}]_{cyt}$ elevation occurring with increasing force (Knight *et al.,* 1992). The magnitude of the elevation correlated with the amount of time the seedling was in motion when responding to wind or touch, and when seedlings were permanently bent no $[Ca^{2+}]_{cyt}$ elevation was measured (Knight *et al.,* 1992). This suggested that the $[Ca^{2+}]_{cyt}$ elevation is only elicited when tension and compression of cells continue to change (Knight *et al.,* 1992). In similar experiments, expulsion of a known volume of air onto a seedling caused a touch response, the magnitude of which increased with rapidity of expulsion (Haley *et al.,* 1995). The effect was also seen in individual cells, as demonstrated by injecting isotonic medium into a suspension of aequorin-expressing tobacco protoplasts (Haley *et al.,* 1995).

The $[Ca^{2+}]_{cyt}$ response to MS appears to occur in all cells and has been observed in the aerial parts (Wright and Knight, in preparation), and in the meristematic, elongation, and differentiated zones and root cap of *Arabidopsis* roots (Legué *et al.,* 1997). The subcellular origins of the Ca^{2+}

signal, however, are still not clear. Unlike the response to cold shock or osmotic stress, the $[Ca^{2+}]_{cyt}$ elevation elicited by touch stimulation cannot be inhibited by the plasma membrane channel blocker La^{3+} or by gadolinium, a stretch-activated calcium channel blocker (Yang and Sachs, 1989), but rather is inhibited by ruthenium red, a putative inhibitor of mitochondrial and ER calcium channels (Knight *et al.,* 1992). A similar lack of effect of La^{3+} or Gd^{3+} was seen in tobacco protoplasts (Haley *et al.,* 1995). Ruthenium red did not, however, affect the touch-induced $[Ca^{2+}]_{cyt}$ elevation elicited in acid-treated epidermal strips in which the only viable cells were guard cells and trichomes (Haley *et al.,* 1995), and in the filamentous moss *Physcomitrella,* EGTA and La^{3+} did cause some reduction of the response, making the interpretation of these data overall difficult.

Attenuation of the touch response by repeated stimulation did not prevent the subsequent elicitation of a normal Ca^{2+} response to cold stimulation, again indicating that Ca^{2+} stores other than the vacuole and the apoplast are involved (Knight *et al.,* 1992). Experiments with plants expressing subcellular targeted aequorin have indicated that the intracellular store involved is unlikely to be the vacuole or the chloroplast (M. R. Knight, 1995, unpublished), and the more likely nucleus, mitochondria, and ER have yet to be explored. An alternative possibility is that after MS, Ca^{2+} is released from Ca^{2+} buffers in the cytosol or that Ca^{2+} bound to the cytoskeleton is released. It is known that microtubule organization has a significant effect on the $[Ca^{2+}]_{cyt}$ changes which occur in response to cold shock and to mechanical stimulation (Mazars *et al.,* 1997). There is increasing evidence that the cytoskeleton may be involved in signaling, with reports of interaction between cytoskeletal components and phosphoinositides. In maize, the actin-binding actin-depolymerizing factor 3 (ADF3) binds to phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate) (PIP_2) and reduces the activity of phospholipase C (PLC) (Gungabissoon *et al.,* 1998). Similarly, the actin-binding protein profilin inhibits plasma membrane PLC in bean leaves (Drøbak *et al.,* 1994).

Determining the origins of the touch-induced $[Ca^{2+}]_{cyt}$ elevation are further confused in the light of other data suggesting a plasma membrane Ca^{2+} channel may be involved in the sensing of MS. The cellular distribution of Ca^{2+} was studied in *Bryonia dioica* after mechanical stimulation, by use of the fluorescent probe CTC which reports membrane-bound Ca^{2+} (Thonat *et al.,* 1993). Cells showed bright fluorescence around the plasma membrane before stimulation, indicating calcium was present in this area of the cell. However, after touch stimulation by rubbing, the fluorescence around the plasma membrane rapidly decreased and was hardly visible at all after 30 sec (Thonat *et al.,* 1993). After a further minute fluorescence had reappeared around the plasma membrane. This change in intracellular Ca^{2+} localization was accompanied by a change in calmodulin location, as deter-

mined by the fluorescence of fluphenazine. Before rubbing, calmodulin-dependent fluorescence was strongly apparent at the periphery of parenchyma cells, but after rubbing it was less intense and less uniformly distributed (Thonat *et al.,* 1993). These data together indicated that MS caused a change in intracellular distribution of both Ca^{2+} and calmodulin and suggested that some of these changes occur in the vicinity of the plasma membrane.

It has been suggested that mechanosensitive calcium-selective channels (MCaCs) in the plasma membrane must play a role in relaying external stimuli (Pickard and Ding, 1993). Using patch clamping techniques, these authors observed that the activity of a plasma membrane MCaC was reduced by treatment with the stretch-activated channel inhibitor gadolinium and that the effect of lanthanides was to briefly increase activity and then to reduce activity (Ding and Pickard, 1993b). The authors suggested that this channel, which also is modulated by electrical, thermal, and chemical factors, may serve as a signal integrator (Pickard and Ding, 1993). Mechanosensitive Ca^{2+} channels have also been reported in the plasma membrane of *Fucus* (Taylor *et al.,* 1997). The fact that a mechanically induced Ca^{2+} channel exists in the plasma membrane, however, does not necessarily mean that this is responsible for the touch-induced elevations of $[Ca^{2+}]_{cyt}$ which have been reported. Such a channel may serve to integrate information regarding mechanical stress with other stimuli which activate it, or activation of the channel may, directly or indirectly, influence the release of Ca^{2+} from other internal stores. An ER-located Ca^{2+}-releasing channel, BCC1, has been identified in tendrils of *Bryonia dioica* and has been shown to be strongly inhibited by the stretch-activated Ca^{2+} channel blocker gadolinium; it therefore provides a possible mechanism for the internal release of Ca^{2+} from a touch-sensitive Ca^{2+} channel (Klüsener *et al.,* 1995). Gadolinium also inhibits tendril coiling in response to touch, and thus one explanation is that activation of this ER Ca^{2+} channel is necessary to transduce the effect of MS to tendril coiling. However, direct evidence that the channel is affected by mechanical stimulation has not been found (Klüsener *et al.,* 1995). In higher plants there is evidence, therefore, that MS can cause activation of a plasma membrane Ca^{2+} channel and that redistribution of intracellular calcium occurs. However, it appears that internal release of Ca^{2+} is required for the MS-induced $[Ca^{2+}]_{cyt}$ elevation.

C. Ca^{2+} Control of Downstream Responses to Mechanical Stimulation

The precise role of Ca^{2+} and calmodulin in thigmomorphogenesis is not clear, but it seems likely that as in the case of abiotic stress responses,

MS-induced $[Ca^{2+}]_{cyt}$ elevations control a number of downstream events including the changes in plant form which occur in response to stimuli such as wind and touch (Knight *et al.,* 1995; Trewavas and Knight, 1994).

Rubbing young *Bryonia* internodes caused an increase in ATP-dependent Ca^{2+} uptake by microsomal vesicles, with the highest rate achieved after 4 hr, followed by a decline in uptake rate after 8 hr (Bourgeade *et al.,* 1991). The effects of protonophores and the inhibitor erythrosine B indicated that the initial increase was likely to be due to stimulation of both a Ca^{2+}-ATPase and a vacuolar H^+/Ca^{2+} antiport (Bourgeade *et al.,* 1991), whereas the later decline corresponded with a reduction in the activity of the antiport (Bourgeade *et al.,* 1991). It was suggested that uptake of Ca^{2+} into microsomes was involved in the reestablishment of Ca^{2+} homeostasis following stimulation. Mechanical stimulation caused an early transient decline in ATP-dependent proton transport followed by a stimulation of transport, lasting an hour (Bourgeade and Boyer, 1994)

Changes in the cellular concentrations of Ca^{2+} and protons are often linked [for example, there is evidence that Ca^{2+} regulates the activity of plasma membrane H^+-ATPase (Kinoshita *et al.,* 1995)], and it is possible that during mechanical stimulation this is also the case. It has been suggested that increase in $[Ca^{2+}]_{cyt}$ may be involved in the initial decline of ATPase activity either by direct inhibition or through a Ca^{2+}-dependent phosphorylation at a control site of the enzyme (Bourgeade and Boyer, 1994). Ca^{2+} may also take part in the recovery and stimulation of ATP-dependent H^+-dependent transport by stimulating a Ca^{2+}-ATPase and an H^+/Ca^{2+} antiport. In maize roots rapid and transient MS-induced activities of vanadate-sensitive K^+,Mg^{2+}-ATPase and the Ca^{2+}-translocating ATPases indicated Ca^{2+} and protons flux across membranes in the early stages of tendril coiling (Liss and Weiler, 1994).

It has been recently reported that Ca^{2+}-regulated kinases and phosphatases are involved in the regulation of actin network tension in soybean cells (Grabski *et al.,* 1998), and evidence has been presented showing that Ca^{2+} transients induce changes in the tension and organization of the actin network through the stimulation of proteins containing calmodulin-like domains or calcium/calmodulin-dependent regulatory proteins (Grabski *et al.,* 1998). Inhibitors of calmodulin-dependent protein kinases caused a significant decrease in tension within the actin network (Grabski *et al.,* 1998) as did the Ca^{2+} ionophore A23187, correlating with rapid decreases in the tension of the actin network observed after A23187 treatment in a previous study (Grabski *et al.,* 1994). The inhibitors calmidazolium and W7, which have been shown to inhibit calmodulin and CDPKs in plants, caused a decrease in resting tension of the actin network (Grabski *et al.,* 1998), whereas incubation in okadaic acid, a phosphatase inhibitor, caused an increase in tension in the actin network (Grabski *et al.,* 1998). More

specific inhibitors of the calmodulin-dependent protein phosphatase 2B (calcineurin) also caused a considerable increase in actin tension (Grabski *et al.,* 1998). In the context of MS signaling, therefore, it is possible that a MS-induced $[Ca^{2+}]_{cyt}$ elevation could stimulate the activity of a CDPK or a Ca^{2+}-independent calmodulin-activated kinase which phosphorylates myosin- or actin-binding proteins leading ultimately to the changes in cell form (and therefore plant form) seen during thigmomorphogenesis.

A number of genes are expressed in response to MS, some of these in a Ca^{2+}-dependent manner (e.g., Braam, 1992a) and others independently of calcium (Botella *et al.,* 1995). Tomato calmodulin mRNA accumulated in response to externally applied 10 m*M* Ca^{2+}, and EGTA had a small effect on the amount and kinetics of accumulation (Depège *et al.,* 1997). Cultured primary *Arabidopsis* root cells exposed to 10 or 100 m*M* Ca^{2+} for 30 min accumulated increased levels of *TCH2, TCH3,* and *TCH4* mRNA although *TCH1* mRNA levels were unaffected (Braam, 1992a). Accumulation began within 10 min of stimulation. *TCH3,4* accumulation kinetics were almost identical in Ca^{2+}-treated root cells or touch-treated plants. Treatment with EGTA caused a decrease in the level of heat shock-induced accumulation of *TCH2* (Braam, 1992a) and similarly cold-shock induced *TCH3* expression (Polisensky and Braam, 1996), although this Ca^{2+} regulation was not demonstrated in response to touch. *TCH3* expression was, however, enhanced by the addition of BAPTA in the absence of a stimulus (Polisensky and Braam, 1996). This may have been due to the fact that chelators can alter Ca^{2+} homeostasis by depleting internal Ca^{2+} stores and resulting in an increase in $[Ca^{2+}]_{cyt}$.

VIII. Interactions between Signals

As mentioned in the introduction to this chapter, there is significant overlap between different forms of abiotic stress and the signaling pathways they trigger. Many stress-inducible genes are expressed in response to more than one stress. For example, *TCH3* is expressed in response to cold and touch (Antosiewicz *et al.,* 1995; Braam and Davis, 1990; Polisensky and Braam, 1996), and a number of genes including *LT178* and *KIN1* are expressed in response to drought and cold (Mäntylä *et al.,* 1995; Nordin *et al.,* 1993). As abiotic stress signaling pathways often converge, there is likely to be ''cross talk'' between pathways, resulting in signaling networks (Jenkins, 1999). Cross talk would provide a mechanism for achieving an integrated and appropriate response to the combinations of stresses which occur in nature. Combinations of stress stimuli may occur concurrently or be temporally separated. In the latter case, plants require some form of ''memory'' in

order to store and retrieve information about previous stress conditions. There are many documented instances of altered responses to stress in plants that have previously experienced similar or different stresses (e.g., Allan *et al.,* 1994; Chen and Li, 1977; Mozafar and Oertli, 1990). The phenomenon of stress acclimation occurs in those cases in which previous exposure increases the ability to survive a subsequent stress challenge. Cold acclimation (described in Section II) results in a gain in freezing tolerance and occurs in response to chilling but can also occur in response to drought stress (Chen and Li, 1977; Siminovitch and Cloutier, 1982). Similarly, in nonhardy species, increased tolerance of nonfreezing temperatures can be induced by chilling (Prasad *et al.,* 1994a), oxidative stress (Prasad *et al.,* 1994b), or osmotic stress (Jennings and Saltveit, 1994). In many cases ''memory'' of the previous stress event is encoded through the expression of protective proteins in response to the first stress episode, and these may go on to confer tolerance of the subsequent stress conditions. For example, genes involved in protection against dehydration stress are expressed in response to drought and produce proteins that may later confer frost tolerance by reducing freeze-induced dehydration. However, other levels of interaction and exchange of information between stress signaling pathways exist. As so many abiotic stress signaling pathways use Ca^{2+} as a second messenger, it remains a possibility that exchange of information between these pathways may occur at the level of Ca^{2+} itself, and evidence has emerged implicating Ca^{2+} in the storage of signals (Verdus *et al.,* 1996, 1997).

Elevations of $[Ca^{2+}]_{cyt}$ can determine whether or not end responses occur, and there is now evidence from studies using animal cells that the shape and the magnitude of the Ca^{2+} transient, the so-called calcium signature, can encode specificity determining which end responses occur (Dolmetsch *et al.,* 1997). The authors demonstrated that when a calcium signature was artificially mimicked, the specific end response could be achieved. In plants a dose dependency has been observed linking the magnitude or strength of stimulus with the size of the $[Ca^{2+}]_{cyt}$ elevation in the cases of touch (Knight *et al.,* 1992), cold (Plieth *et al.,* 1999), salinity, drought (Knight *et al.,* 1997), and ozone (Clayton *et al.,* 1999). It is possible, therefore, that one way of specifying altered end responses in plants which have been previously stressed could be via altering the calcium signature.

Experiments with aequorin-expressing *Arabidopsis* have shown that the kinetics and magnitude of abiotic stress-induced calcium signature do change depending on the previous stress history of the plant (Knight *et al.,* 1996, 1998). Plants acclimate to drought conditions after previous experience of drought (Levitt, 1986; Siminovitch and Cloutier, 1982). Drought acclimation (administered by mannitol pretreatment), caused seedlings to respond to mannitol treatment on subsequent days with a greater than normal $[Ca^{2+}]_{cyt}$ elevation (Knight *et al.,* 1998). This increased elevation was

accompanied by an increase in the magnitude of *AtP5CS1* gene expression (Knight *et al.,* 1998) and an increased capacity to survive the drought stress treatment. It could be envisaged that increased expression of a signaling component, such as PLC (see Section III.A), may be amplifying the subsequent Ca^{2+} signal to cause a greater downstream effect on gene expression (Knight *et al.,* 1998). Sensitivity to low temperature treatment can, in a similar way, be increased by previous exposure to oxidative stress. In *Arabidopsis* plants pretreated with H_2O_2, cooling-induced $[Ca^{2+}]_{cyt}$ elevations could be elicited by less severe reductions in temperature than those required to elicit such elevations in control plants (Knight and Knight unpublished; see Fig. 2A; see color insert). Other stress pretreatments have effects on stress-induced Ca^{2+} signaling and cause either increases or decreases in the magnitude of the subsequent $[Ca^{2+}]_{cyt}$ elevation, the effect depending on the combination of stresses used (Fig. 2B; see color insert). Some stress combinations can result in decreased downstream response to later stress. For example, oxidative stress pretreatment (by application of H_2O_2) resulted in a much reduced $[Ca^{2+}]_{cyt}$ elevation in response to mannitol and a corresponding reduction in the levels of mannitol-induced *AtP5CS1* expression (Knight *et al.,* 1998). It appears throughout these studies that abiotic stress-induced Ca^{2+} signatures reflect both the current stimulus and previous conditions and that these may provide a mechanism for achieving appropriate integrated responses.

IX. Concluding Remarks

The previous sections have demonstrated that cellular Ca^{2+} levels are elevated in response to a variety of forms of abiotic stress. Evidence has also been presented to show that these $[Ca^{2+}]_{cyt}$ elevations proceed to control downstream end responses through their effects on calmodulin, CDPKs, and other proteins and eventually to control gene expression. The big question is how is specificity of response encoded and controlled? A number of possibilities exist, and it is quite possible that specificity of response relies on one or more of these.

A. Cell-Specific Responses

Some stimuli may elicit $[Ca^{2+}]_{cyt}$ changes only in particular cells, especially those for which the stimulus has a particular physiological relevance, for instance, the root for changes in moisture content in the soil, or stomatal guard cells for changes in CO_2 levels. To date few reported examples exist;

however, this must on part be due to the experimental difficulties involved in making $[Ca^{2+}]_{cyt}$ measurements in more than one cell type. The study of cell-specific Ca^{2+} signaling will be aided by the production of plants expressing cell-specific recombinant Ca^{2+} indicators in the same way that cell specific GFP-expressing lines have been produced (Haseloff, 1999). Some of these are already being developed (A. Haley, personal communication). Once such resources are available, Ca^{2+} responses to stimuli can be recharacterized and the role of cell specificity assessed.

B. Intracellular Source

Specificity may also be encoded via the intracellular source of the $[Ca^{2+}]_{cyt}$ elevation. The vacuole (Knight *et al.,* 1996, 1997) and mitochondria (Subbaiah *et al.,* 1998) have been shown to contribute to abiotic stress-induced $[Ca^{2+}]_{cyt}$ elevations, and it is likely that the ER functions as a Ca^{2+} store in a similar manner. [Other stimuli may elicit changes in Ca^{2+} levels *within* an organelle, such as the chloroplast (Johnson *et al.,* 1995) or the nucleus.] Targeting of aequorin is in progress and may be of use in identifying the intracellular store involved in the touch-induced $[Ca^{2+}]_{cyt}$ elevation. The overall importance of subcellular origins of Ca^{2+} has yet to be fully determined; however, it does appear that subcellular store alone would be insufficient to entirely account for specificity as so many different stresses appear to involve an increase in $[Ca^{2+}]_{cyt}$ due to influx across the plasma membrane (e.g., Knight *et al.,* 1996, 1997; Price *et al.,* 1994). A further level could exist in encoding specificity through a dependency on which particular Ca^{2+} channels are involved. Therefore many stimuli may elicit Ca^{2+} influx but via different plasma membrane Ca^{2+} channels or different combinations of channels. This idea is supported by the evidence that many responses which appear to involve influx of Ca^{2+} are not inhibited by the same Ca^{2+} channel blockers. The use of different Ca^{2+} channels (at either the same or a different subcellular store) might also achieve specificity through the use of different downstream effector proteins located in the vicinity of different channels.

C. Involvement of Other Signaling Components

In order to achieve downstream responses, Ca^{2+} must interact with other proteins, and its effect may also be modified by other second messengers. Specificity of response to particular stresses could be achieved by the necessity for an additional component, which is produced only in response to one specific stress. Cyclic nucleotides have been reported as antagonizing

Ca^{2+} dependent pathways (Bowler *et al.,* 1994) and can themselves elevate cytosolic Ca^{2+} (Volotovski *et al.,* 1998). It is possible therefore that cyclic nucleotide levels are altered in response to particular stresses and mediate specific responses to these stresses either by modifying the calcium signature or by affecting Ca^{2+}-dependent pathways downstream of calcium.

D. Specificity through Ca^{2+} Signature

As discussed above, the calcium signature may be a prime component in determining the specificity of end response. It has been suggested that the magnitude and kinetics of the $[Ca^{2+}]_{cyt}$ elevation encode information which influences the choice of final response. Certainly this has been shown to be true in animal cells (Dolmetsch *et al.,* 1997; see above). Many factors could potentially influence the stress-induced Ca^{2+} signature which occurs in a particular cell type, including the subcellular source of Ca^{2+}, the subpopulation of Ca^{2+} channel employed, and possibly the interaction of other factors such as cyclic nucleotides (as listed above). It is still not known what features of the Ca^{2+} signature may be the most important in determining specificity of response, although (as mentioned in Section VIII) correlations have been seen between the size of certain parts of the signature and the magnitude of the stimulus applied (H. Knight and M. R. Knight, 1997, unpublished results; Plieth *et al.,* 1999; Clayton *et al.,* 1999; Knight *et al.,* 1992). One parameter that has been suggested as determining downstream physiological events is $[Ca^{2+}]_{cyt} \times$ time. If this were the case, the characteristics of the end response could be determined by the magnitude and duration of the response kinetics (Malhó *et al.,* 1998). Similarly, specificity may be encoded in the periodicity or magnitude of oscillating Ca^{2+} spikes (Hetherington *et al.,* 1998; Malhó *et al.,* 1998; McAinsh *et al.,* 1995). The shape and timing of Ca^{2+} oscillations differ with the nature of the stimulus (Hetherington *et al.,* 1998).

In summary, the underlying mechanisms encoding specificity are at present poorly understood in plants and will require new tools, such as specific recombinant indicators and methods for artificially producing Ca^{2+} signatures "to order." A greater understanding of these processes will be required before plant responses to abiotic stress can be manipulated and specifically improved by altering Ca^{2+} signaling.

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