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Kwang W. Jeon

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

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Front cover paragraph: Ptk2 cells stained with anti-tubulin antibodies. (For more details, see Chapter 2, Figure 3b.)

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CONTRIBUTORS

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

Arnold De Loof (249), *Laboratory for Developmental Biology and Molecular Biology, Zoological Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Sher Karki (69), *Cell and Molecular Biology Graduate Group, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Elizabeth A. Holleran (69), *Cell and Molecular Biology Graduate Group, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Erika L. F. Holzbaur (69), *Department of Animal Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Dina F. Mandoli (1), *Department of Botany, University of Washington, Seattle, Washington 98195*

Liliane Schoofs (249), *Laboratory for Developmental Biology and Molecular Biology, Zoological Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Zohreh Tabaeizadeh (193), *Department of Biological Sciences , University of Quebec in Montreal, Montreal, Quebec H3C-3P8, Canada*

Joseph A. Trapani (111), *The John Connell Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg 3084, Australia*

Dirk Veelaert (249), *Laboratory for Developmental Biology and Molecular Biology, Zoological Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

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What Ever Happened to *Acetabularia?* Bringing a Once-Classic Model System into the Age of Molecular Genetics[1]

Dina F. Mandoli
Department of Botany, University of Washington, Seattle, Washington 98195

In the 1930s *Acetabularia acetabulum* made major contributions to understanding how the genomes in a cell coregulate development and morphogenesis. Unique and elegant experiments were possible because of two features of this marine alga: it is a large unicell that withstands amputation and intra- and interspecific grafting and it has only one nucleus (until it reproduces) which resides in the base of the organism. At a time when the role of nucleic acids was unclear and decades before their chemical nature was defined, these features of *Acetabularia* enabled the relative contributions of the nucleus and the cytoplasm to growth, development, and morphogenesis to be distinguished. In addition, grafting and amputation phenomena suggested the existence of mRNA nearly 30 years before its discovery. This review documents the decline in use of the system, justifies why it is well worth resuscitating, and summarizes advances in methodology that make that renaissance possible.

KEY WORDS: *Acetabularia acetabulum,* Dasycladales, Culture methods, Life cycle duration.

I. Introduction

The protist *Acetabularia acetabulum* (a.k.a. *Acetabularia mediterranea*) is a model organism which holds a classic place in the literature and in textbooks (Taiz and Zeiger, 1991). Like other model organisms, such as *Drosophila, Arabidopsis,* and *Saccharomyces, A. acetabulum* offers unique ap-

[1] The author dedicates this review to Brenda E. Hunt for her many years of fine technical support on the occasion of her leaving research. This research certainly would have been less fun, perhaps not even possible, without her.

proaches to problems in biology that are of broad relevance, such as the role of localized determinants in development and morphogenesis. It is a powerful model organism for two reasons: it is a giant, unicellular plant which has a single nucleus for most of its life cycle yet it has complex cytoarchitecture and development, and it lends itself to a wide range of experimental techniques. Despite its obvious potential to advance understanding of development, it is not widely used today.

This review addresses why this system fell into disuse and seeks to establish arguments as to why it is worth resuscitating. To illustrate the power and unique attributes of the organism, some of the seminal older experiments are briefly revisited. A synopsis is provided of the arenas to which the system stands to contribute to current biological frontiers. Next, the biological and technical pros and cons of each portion of the development of the organism are assessed, in the hope that frank appraisal will spur resolution of the few remaining pitfalls. Finally, an appendix of methods that will facilitate access to the system is compiled with special attention to recent advances in fast, efficient culture techniques. This review makes no attempt to discuss the voluminous literature on *A. acetabulum* and the many related species. Bibliographies that span the years 1640–1980 are available in hard copy (Bonotto and Lüttke, 1980; Schweiger *et al.,* 1974). The reader is referred to additional reviews on the general biology (Berger *et al.,* 1987; Bonotto, 1988; Puiseux-Dao, 1962), evolution, phylogeny, and geographic distribution (Berger and Kaever, 1992), development (Hämmerling, 1953, 1963; Mandoli, 1996; Menzel, 1994), organelles (Lüttke and Bonotto, 1982), circadian rhythms (VanDen Driessche *et al.,* 1997), electrophysiology (Gradmann, 1984), and cytoskeletal components (Menzel, 1994) of the family and genus.

A. About the Organism

Acetabulariaceae is one of two families of subtropical and tropical green algae in the marine order Dasycladales (Berger and Kaever, 1992). About 11 genera of Dasycladales are extant but at least 186 genera are recognized today when fossil genera are included (Berger and Kaever, 1992; Bold and Wynne, 1985). This successful group has a history that began in the Cambrian, 570 million years ago, and has a strong fossil record (Berger and Kaever, 1992) because they calcify in the wild (Marin and Ros, 1992). Based on morphological data (Berger and Kaever, 1992) and rDNA sequences (Olsen and Stam, 1994), the Dasycladales are not considered in the lineage of green algae which led to the evolution of land plants. Although the geographic distribution of the extant family has been well documented (Berger and Kaever, 1992), their ecology has been sparsely

described (Berger and Kaever, 1992; Marin and Ros, 1992). Three Dasycladales that have been most well studied developmentally are two Mediterranean, *A. acetabulum* and *A. crenulata,* and a Japanese species, *A. calyculus.* This review focuses on the type species *A. acetabulum.*

A. acetabulum reaches 2–4 cm tall at reproductive maturity when grown in the laboratory. This species has a complex architecture (Fig. 1) and follows an elaborate developmental program during each life cycle (Fig. 2). In brief, growth of the vegetative stalk occurs mainly along one axis. Stalk growth is periodically interrupted by initiation of a whorl of hairs which encircle the stalk. The hairs are extensions of the stalk wall which ramify as they grow (Fig. 1) and contain variable numbers of chloroplasts depending on when in development they form (Table II). A single nucleus (Berger *et al.,* 1987; De and Berger, 1990) resides in the basal rhizoid of this alga (Fig. 1). Vegetative growth is composed of three phases (juvenile, early, and late adult) which are temporally sequential and spatially stacked, differ in development behaviors (Runft and Mandoli, 1996) and physiological responses (Zeller and Mandoli, 1993), and can be distinguished by gross

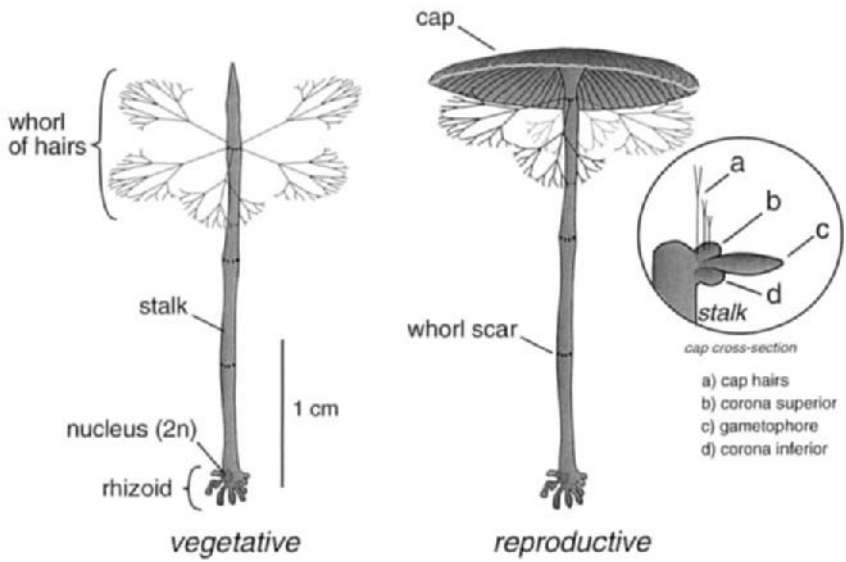

FIG. 1 Gross anatomical features of vegetative and reproductive plants of *A. acetabulum* are shown to approximate scale (see the 1-cm vertical bar). The inset shows a cross section of the reproductive structure, the cap. Only a few whorls and whorl scars are shown for the sake of clarity (see text for the correct values). Whorls of hairs which emerge from the superior corona of the cap (see inset) have been omitted from the cartoon depicting the reproductive plant. The number of sterile hairs per whorl has also been reduced for clarity.

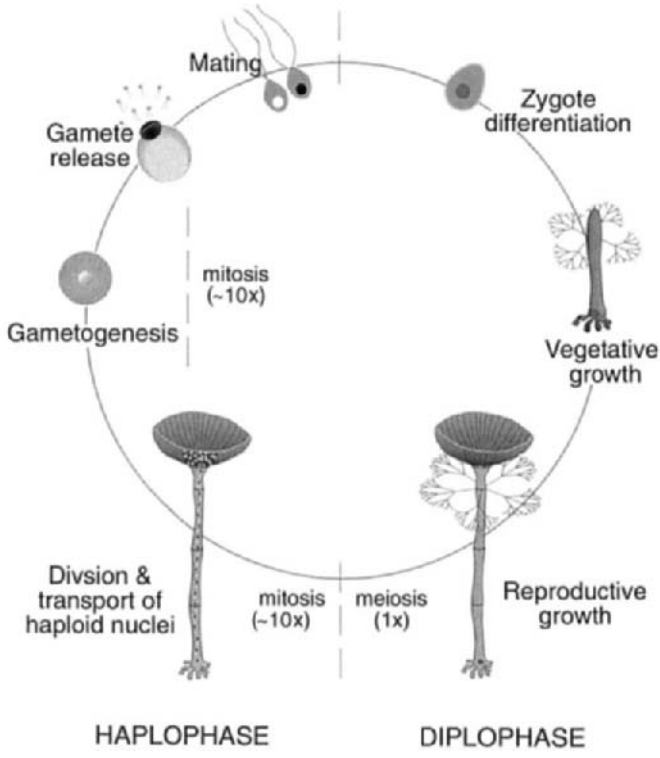

FIG. 2 The basics of the haploid and diploid portions of the life cycle of *A. acetabulum*. Major portions of the life cycle that comprise a unit of development, i.e., all of gametogenesis, are united by an arc subtending the relevant label. For the sake of clarity, cartoons of the organism are not to scale. Additional details about each portion the life cycle, including the relevant sizes of each portion of the life cycle, can be found in the text.

morphology (Nishimura and Mandoli, 1992b). Vegetative growth ends with initiation of a disc-shaped "cap" at the stalk apex (Fig. 1). During reproduction, an individual resembles an open, green umbrella consisting of a rhizoid, stalk, and cap.

The nuclear cycle of the organism is also complex. At cap initiation the alga is uninucleate and unicellular (Fig. 1, Table I). During cap expansion, the nucleus undergoes one round of meiosis (Runft and Mandoli, 1996). Multiple mitotic divisions ensue, generating thousands of haploid "secondary" nuclei per individual (Table I). The secondary nuclei and the cytoplasm from the vegetative portions of the alga migrate through the stalk up into the gametangial rays. Once there, each nucleus and some cytoplasm are enclosed together by a new gametangial wall. Additional rounds of mitosis

TABLE I
Types of Nuclei during the Life Cycle of *Acetabularia acetabulum*

	Ploidy	Location	Number per individual	References
Primary	2n	Rhizoid	1	De and Berger (1990); Green (1973); Hämmerling (1932); Koop (1975a); Spring *et al.* (1978)
Secondary	n	Stalk and cap	4.4 thousand	Nishimura and Mandoli (1992a)
Tertiary	n	Gametangia	2–6 million	Geil and Mandoli (1998a); Green (1973); Koop (1975a); Mandoli and Larsen (1993)

occur inside the nascent gametangia, generating thousands of haploid "tertiary" nuclei per gametangium (Table I). Since a gametangium originally enclosed one haploid nucleus, each gametangium makes gametes of one mating type (see references in Table I; Geil and Mandoli, 1998a). The life cycle is completed by release of isogamous gametes from mature gametangia, followed by mating and growth of the diploid zygote into a young, vegetative organism. The alga is prolific, producing millions of progeny per plant per generation (Table I) and can be self- and out-crossed.

B. What Made *Acetabularia* a "Classic" Developmental System?

This marine alga offers unique avenues of attack to current problems in biology. First, because it is physically 10–10,000 times larger than the cells of multicellular plants and animals, parts of the organism can be manipulated via amputation, grafting (Hämmerling, 1963; Mandoli and Hunt, 1996), and nuclear transplants (Hämmerling, 1934b, 1963). Second, because it is a unicell, subcellular interactions during development (e.g., biology of the putative "reproduction-specific" mRNAs) can be addressed without the complication of cell–cell interactions. Third, the environmental cue, light, which regulates vegetative and reproductive development (Kratz and Mandoli, 1998a; Schmid *et al.*, 1987), can be applied cleanly and quantitatively and withdrawn rapidly ($\leq \mu$s). Finally, the alga is amenable to classical and molecular genetics including transformation (Cairns *et al.*, 1978; Geil and Mandoli, 1998b; Neuhaus *et al.*, 1984, 1986), as well as biochemical (proteins: Bannwarth and de Groot, 1991; Bannwarth *et al.*, 1982; Bannwarth and Schweiger, 1983; Moritani *et al.*, 1997; nucleic acids: Yang and

de Groot, 1992; Yang and Scheid, 1992) and whole-organism manipulation (Berger *et al.,* 1987; Hämmerling, 1963; Mandoli and Hunt, 1996). This suite of technical and biological features makes this organism extremely attractive to developmental biologists.

Many fine researchers have contributed important methods and results to the literature. However, for the purposes of this review and for the sake of brevity, touching on just three periods in the research history of *Acetabularia* will suffice to highlight a few arenas to which the system has made significant contributions to the understanding of developmental biology.

In broad terms, Joachim Hämmerling's contributions from 1931 to 1966 were to make the system experimentally tractable for cell-biological studies and to use its unique features to examine the interactions between the nucleus and the cytoplasm. Apparently, as a postdoctoral fellow with Hartmann (Berger and Kaever, 1992), he accomplished the difficult task of domesticating the alga to laboratory culture by fully documenting the anatomy and life cycle of the organism (Hämmerling, 1931). In a body of now classical experiments, he began to distinguish the contributions of the nucleus and the cytoplasm to morphogenesis and development by amputating the nucleus-containing region from the rest of the organism. In other words, he bisected the nucleate rhizoid from the enucleate apex. Recall that it was the 1930s and the role of the nucleus, much less the chemical nature of DNA, was not known. Surprisingly, the nucleus was superfluous: the apex completed morphogenesis, making whorls and a cap, without the nucleus (Hämmerling, 1932, 1934b). When the organism was cut into three sections, a rhizoid, a midsection, and an apex, the rhizoid could complete development, the enucleate midsection could make whorls but failed to make a cap, and the apex could make both whorls and a cap (Hämmerling, 1934b, 1936). Based on these dissections he concluded that there was a gradient of information, of "morphogenetic substances," from the base to the apex of the organism that dictated the morphogenetic capacity of each region of the species. Having established the fact that the midsection was incapable of cap morphogenesis on its own, and reasoning that the information to make a cap must be coming from somewhere, he delayed amputation of the rhizoid for a few days to show that the nucleus could render the midsection competent (Hämmerling, 1934b). Further proof that the nucleus was the source of the morphogenetic substances for the cap came from interspecific grafts in which the cytoplasm of a species which made a mop-shaped cap was joined to the rhizoid of a species which made a cup-shaped cap. Each such graft chimera made multiple caps in succession which differed in morphology. Interspecific graft chimeras initially made a cap consistent with the morphology of the apical donor, then formed a cap with morphological features of both species, and finally made a cap consis-

tent with the morphology of the rhizoidal donor. Hämmerling interpreted these data to mean that the morphogenetic substances from different species could be coexpressed but were used up during morphogenesis so that the shape of the cap ultimately depended only on the nuclear donor (Hämmerling, 1934b, 1963). Perhaps Hämmerling's most important intellectual contribution was the recognition of the power of a large unicell with a single nucleus to study the interaction between the nucleus and the cytoplasm during development and morphogenesis and his finest experimental accomplishment was rendering the Dasycladaceae experimentally tractable.

Apparently inspired by Hämmerling (Berger and Kaever, 1992), from 1951 to 1989 Jean Brachet studied several types of unicellular animals, plants, and oocytes, seeking to understand how the nucleus and cytoplasm interact during development. In the 1950s, a time when the autonomy of organelles was not known, his lab showed that chloroplasts isolated from anucleate apices incorporate labeled amino acids into their own proteins and helped provide evidence that the cytoplasm had DNA of its own (Brachet, 1981). To discern the chemical nature of the "morphogenetic substances" that were proposed to exist in *A. acetabulum* by Hämmerling, he and his colleagues used exogenous inhibitors of organellar and nuclear transcription and translation (Bonotto *et al.,* 1968; Brachet, 1968; Brachet *et al.,* 1964), reducing agents (Brachet and Lang, 1965), and localized UV irradiations in the presence of radioactive methionine or adenine (Brachet and Olszewska, 1960; Olszewska and Brachet, 1961). Based on his findings, in a 1959 address (Brachet, 1960), he suggested that the Hämmerling's "morphogenetic substances" were stable mRNAs (Brachet, 1981). The next year, Jacob and Monod (1961) published evidence for the role of mRNA in prokaryotes. Brachet's fascination with how the "tridimensional structure of the cap" was formed (Brachet, 1970), the role of mRNAs in morphogenesis (Brachet, 1960; Farber *et al.,* 1968), and with unicells such as *Acetabularia* and amphibian oocytes clearly anticipated current interest in how localized determinants orchestrate development and morphogenesis.

Two contributions of Hans-Georg Schweiger's laboratory from 1960 to 1986 were to add molecular techniques to the search for the "morphogenetic substances" and to distinguish the roles of the nucleus and cytoplasm to the circadian rhythms of the alga. His laboratory studied polyadenylated RNA stability and synthesis (Kloppstech and Schweiger, 1975, 1976, 1977, 1982) and developed stable transformation in *Acetabularia* (Neuhaus *et al.,* 1986). Transformation with foreign DNA, novel at the time, remains remarkable because such a wide variety of promoters and genes functioned, because it was 70% effective, and because the foreign DNA was stably inherited by the progeny for at least three generations (Neuhaus *et al.,* 1986). Since the 1980s transformation via microinjection of the nucleus has not been further characterized in this species. Two overt circadian rhythms

of the alga are photosynthetic output (Schweiger *et al.*, 1964) and chloroplast migration (Koop *et al.*, 1978). The photosynthetic output of this species has a maximum at noon and, as expected, anticipates dawn (Schweiger *et al.*, 1964; VanDen Driessche *et al.*, 1997). To see where the "clock" resided, Schweiger made graft chimeras between algae that had been grown on light:dark cycles that were 180 degrees out of phase. In other words, he took two individuals grown on opposite light:dark cycles and swapped their rhizoids. When the graft chimeras were then grown in continuous light, i.e., in the absence of an environmental diurnal cue, and oxygen evolution of a few grafts was measured. The results suggested that the rhythm of the graft chimeras followed the entrainment of the rhizoid donor. To ask if the rhythm was conferred by the nucleus itself or by the rhizoidal cytoplasm, the experiment was repeated but now only the nucleus was implanted into an enucleate partner. These graft chimeras followed the prior entrainment pattern of the nuclear donor, indicating that the nucleus, not the cytoplasm surrounding the rhizoid, carried out the pacemaker function of the alga. Note, however, that similar experiments on chloroplast movement did not confirm the role of the nucleus in setting the phase of this rhythm (Woolum, 1991). Using a molecular approach to identify genes that regulated circadian rhythms, Schweiger's group found sequences in *A. acetabulum* homologous to the *Drosophila* gene *period* that paradoxically were located in the chloroplast genome (Hartwig *et al.*, 1985, 1986; Li-Weber *et al.*, 1987; Schweiger *et al.*, 1986a). *period,* the only defect known to knock out all overt rhythms when it is deleted, has the phenotype expected of a defect in the central pacemaker. However, despite many homologies to *per* in species outside of the drosophilids, no functional significance of the region studied in *A. acetabulum* has been demonstrated in any organism (Kyriacou *et al.*, 1996). A different conserved sequence, called PAS, is now recognized as more telling of circadian function (Crosthwaite *et al.*, 1997). In sum, Schweiger capitalized on the unique experimental tricks to which *A. acetabulum* lends itself and his insightful application of molecular tools was on the leading edge of the molecular era.

C. Why *Acetabularia* Remains an Attractive System

How the spatial and temporal regulation of development occurs when the nucleus and the site of differentiation are so remote from each other underlies many of the central questions that may be uniquely addressed in *A. acetabulum.* A few examples of the interesting aspects of the alga illustrate the potential of the system.

- The nucleus and the major site of morphogenesis, the stalk apex, coordinate development several times during the life cycle. As in nerves,

this crosstalk is remarkable because the nucleus and stalk apex are at opposite ends of the organism, 2–4 cm apart. (In another species, *A. major,* the nucleus and apex are 10 cm distant.) Temporal and spatial coordination between the nucleus and the apex is especially critical during reproduction: a unicell with a single nucleus which donates all of its body contents to its progeny cannot afford to make an error. For example, meiosis usually ensues after a cap has initiated, but if a cap aborts and vegetative growth resumes, it was inferred that meiosis is also delayed because no secondary nuclei appear (Hämmerling, 1963). These data suggest that the nucleus and apex alter the timing of reproduction if one or the other is not ready. Later in reproduction, the contents of the parental algal body and thousands of haploid nuclei are spatially arranged together and then cotransported from the rhizoid up into the cap (Fig. 2). Virtually nothing is known about the signal transduction inherent in the long-distance communications between the nucleus and the apex.

- The cell cycle of this alga is unusual in that karyokinesis and cytokinesis are not obligately linked. The single diploid nucleus undergoes one round of meiosis, and then the haploid products of meiosis undergo multiple rounds of mitosis to generate a syncytium within the parent body. Later in development, this population of haploid nuclei undergoes more rounds of karyokinesis during gametogenesis (Fig. 1, Table I). Calculations indicate that the average individual in a heterogeneous wild-type strain undergoes ~10 rounds of mitosis during each of these two mitotic bursts (Mandoli and Larsen, 1993; Nishimura and Mandoli, 1992a). While other organisms have unusual cell cycles (Bold and Wynne, 1985), few offer the range of experimental manipulations, e.g., with grafting, transformation, and classical genetics, which enables study of both nuclear and organellar genomes during a cell cycle in which cytokinesis and karyokinesis are uncoupled.
- For biochemical studies, the alga may provide a rich source of proteins and RNAs involved in DNA synthesis because it makes millions of haploid nuclei theoretically producing an average of 6.5 μg of DNA per organism per generation [calculation is based on 0.92 pg of DNA per haploid genome (Spring *et al.,* 1978) $\times$ 1.6 $\times$ 10^3 gametes per gametangium (Mandoli and Larsen, 1993) $\times$ 4.4 $\times$ 10^3 gametangia per individual (Nishimura and Mandoli, 1992a)]. In addition, the alga may prove a source of genes unique to the marine environs, such as a plasma-membrane-bound Cl^--ATPase (Moritani *et al.,* 1997), which is a major anion pump in this organism. Unlike most unicells, which do not lend themselves to biochemical studies, *Acetabularia* are so large that assays require as few as 4 algae for an assay for DNA biosynthetic enzymes (Bannwarth and de Groot, 1991; Bannwarth *et al.,*

1982; Bannwarth and Schweiger, 1983) and isolation of most of the contents of the alga is as trivial as cutting off one end and squeezing out the cytoplasm just as one removes the contents of a tube.

- The cytoskeleton is actively involved in vegetative and reproductive development (Menzel, 1994). The cytoskeleton has a clear role in moving nuclei from the rhizoid to the apex of the organism (Menzel, 1986) and in partitioning of the cytoplasm during gametogenesis (Menzel and Elsner-Menzel, 1990). The cytoskeleton moves cytoplasm during streaming. During the day, the chloroplasts stream actively through the stalk and rhizoid but at dusk they migrate into the basal rhizoid of the alga near the nucleus where they remain for the night (Koop *et al.,* 1978). Additional roles of the cytoskeleton in coordinating organismal functions await exploration.
- Molecular evolution of this ancient organism has just begun to be tapped (Olsen and Stam, 1994). A good example is the interaction between the chloroplast and nuclear genomes, which has yet to have been resolved satisfactorily. The chloroplast genome size (i.e., the amount of DNA) is among the largest known and, perhaps consistent with this, the chloroplast has retained DNA synthesis functions that in most plants are nuclear (Bannwarth and de Groot, 1991; Bannwarth *et al.,* 1982). Originally, restriction analysis suggested that the chloroplast genome may be as large as 400 kb (Schweiger *et al.,* 1986b; Woodcock and Bogorad, 1970) and cesium gradients and kinetic analysis both suggest that the chloroplast genome is complex (Green, 1973; Padmanabhan and Green, 1978). Two pieces of evidence suggest that the chloroplast genome is variable, however, and this may have skewed estimates of its size. First, Southern analyses with several probes showed length polymorphism between distinct geographic isolates (Leible *et al.,* 1989; Tymms and Schweiger, 1989), raising the possibility that the nature of the symbiotic interaction between the nuclear and chloroplast genomes varied between isolates. Second, only 20–50% of the chloroplasts contain DNA which can be visualized with stains and electron microscopy (Lüttke, 1988; Lüttke and Bonotto, 1981; Woodcock and Bogorad, 1970), suggesting that the chloroplasts in each individual may not have the same DNA content (Green, 1973). [Parenthetically, if the majority of chloroplasts lacked DNA it was hard to explain the high gamete viability (Mandoli and Larsen, 1993) because there is only one chloroplast per gamete (Crawley, 1966, 1970; Herth *et al.,* 1981). The percentage of the plastid population that has a genome stainable with 4′,6-diamidino-2-phenylindole (DAPI) was a function of developmental age (Table 1 in Lüttke and Bonotto, 1981). When the organelles of *A. cliftonii* were stained with the fluorescent dye $DiOC_6$, confocal microscopy suggested that the chloroplasts in this species are interconnected

(Menzel, 1994). While the literature on chloroplast DNA content was not raised, these data clearly suggest that the puzzling results on the DNA content of chloroplasts of *A. acetabulum* were an artifact derived from interpreting the chloroplasts *in vivo* as discrete units when they were actually not. Interestingly, tubules that bridge chloroplasts which are similar in appearance and length to those found in *A. acetabulum* have been visualized in several land plants by expressing a green fluorescent protein targeted to the chloroplast by a *rec*A transit peptide (Köhler *et al.,* 1997). These data suggest that *in vivo* chloroplasts in several taxa may not always be functionally isolated organelles.] Other questions of molecular evolution, such as the molecular basis of the evolution of a discrete reproductive from sterile whorls, which split the order into the Dasycladaceae and the Acetabulariaceae, may be facilitated by the 570-million-year fossil record (Berger and Kaever, 1992).

- Haploid-specific gene expression may occur in *A. acetabulum.* For example, cellulose wall polymers enclose the gametangia whereas mannan-based polymers predominate in the sporophyte walls in which cellulose is either absent or present at low levels (Herth *et al.,* 1975; Kline *et al.,* 1997). This difference in wall composition of the haplophase and the diplophase also occurs in the marine coenocyte *Derbesia* and in *Bryopsis* (Codiales) (Huizing and Rietma, 1975; Rietma, 1973). Gametes have unique structures such as the eyespot and the flagellae, but whether these gametophytic structures are both made and assembled or only assembled during the haplophase is unknown.
- *A. acetabulum* may be the only organism in which it is possible to drive a phenotype arrested in vegetative growth all the way through normal development with wild-type cytoplasm alone (Fig. 3). In other systems such as *Danio,* or in *Drosophila* and *Xenopus* oocytes, one can only locally rescue phenotypes or drive development a bit further. The *A. acetabulum* defect *kurkku* (*kur*) arrests in juvenile growth (Mandoli and Hunt, 1996) and segregates as a single Mendelian trait (D. F. Mandoli and B. E. Hunt, unpublished observations). *kur* rhizoids complete reproduction when grafted to enucleate apices from wild type (Mandoli and Hunt, 1996). Such grafts produce viable gametangia whose progeny on self-cross are all *kur* (B. E. Hunt and D. F. Mandoli, unpublished observations). The added access to classical genetics with the graft progeny of *A. acetabulum* is a powerful combination of tools for the study of development.

This incomplete list does not attempt to order the significance of the topics in any way but does begin to illustrate the power and flexibility of the system. What is known about the relationships of extinct to extant

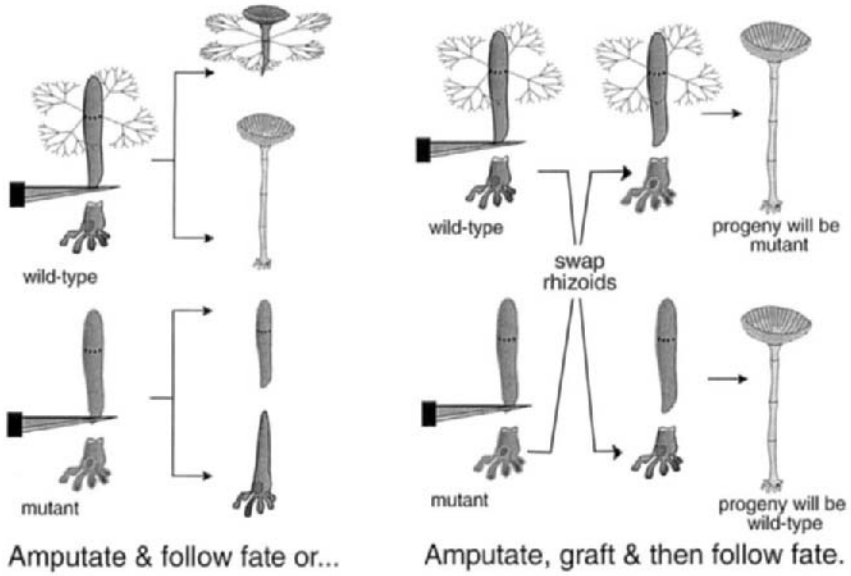

FIG. 3 Responses of *A. acetabulum* to amputation and grafting. The vertically aligned cartoons are to scale with each other, but horizontally aligned cartoons are not necessarily at the same scale. (Left) Typical fates of the enucleate apex and the nucleated rhizoid to amputation are shown for wild type and for a Class I type defect which is arrested in development, e.g., *kurkku* (Mandoli and Hunt, (1996). (Right) The fates of graft chimeras of a reciprocal graft in which the rhizoids of a wild-type and mutant plant are exchanged. Progeny of these graft chimeras are shown. This depiction may also be valid for some other species and for some combinations of interspecific graft chimeras.

species is clearly explained in a thorough and beautifully illustrated monograph (Berger and Kaever, 1992). The ability to analyze development using a multifarious approach, that is, a combination of biological, biochemical, molecular, and now, genetic methods, means that *A. acetabulum* remains an exciting unicellular organism for the study of development.

D. History of the Rise and Fall of *Acetabularia*

The publication record of a system is one way to document its importance and use (Fig. 4; Schweiger *et al.,* 1974). The organism was first drawn in 1586 (Mattoli, 1586), but it was 54 years before the second (Parkinson, 1640) and 172 years before the third publication (Linneaus, 1758) appeared

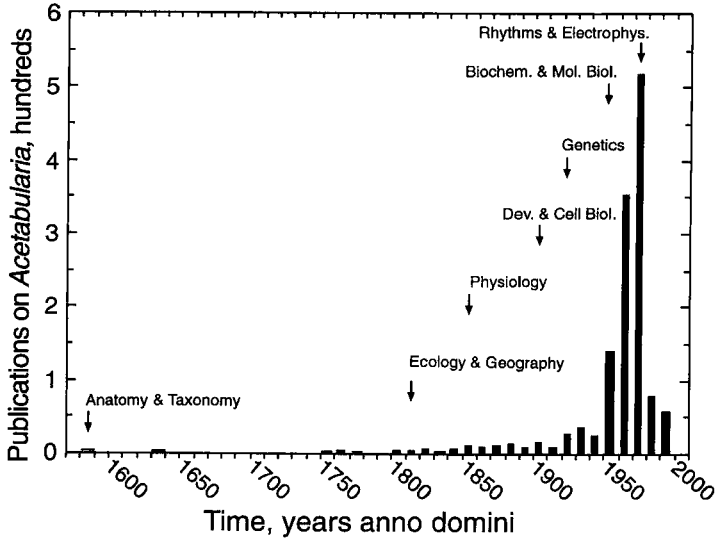

FIG. 4 History of publication for *Acetabularia* based on Bonotto and Lüttke (1980), Schweiger *et al.* (1974), and commercial compilations of the more recent literature (i.e., Current Contents, Biosis, and Medline). The numbers of publications per decade are graphed from the first publication in 1586 to 1997. General topics of publication are marked. Arrows mark the first publication of a new type and are labeled accordingly.

(Fig. 4). As perhaps is typical, the focus was on anatomy and taxonomy initially and was on physiology and molecular biology later (Fig. 4). The rise of this system followed closely after the discovery in the 1930s by Hämmerling that the alga had just one nucleus. The first review on the organism, one sign that a system or topic is significant to the larger scientific community, was published in 1952 (Brachet, 1952a,b), when 162 publications had accumulated. The rapid rise in publication frequency in the 30 years from 1950 to 1980 (Fig. 4) reflects the historical rise in science and speaks to the utility of the system in addressing the salient biological questions of the day, namely, elucidation of the role of the nucleus and cytoplasm in orchestrating basic biological functions.

However, the number of publications since 1980 to the present has declined as precipitous as the rise that brought the system to prominence (Fig. 4). The trend in the U.S. from 1980 to 1997 to support just a few model systems has certainly contributed to this pattern and this pattern of publication may be typical of many organisms. In the case of *Acetabularia,* the untimely death of Schweiger in 1986 forced the contraction of a large center of research in Germany (Max-Planck-Instituts are closed when the

leader retires or moves on) and so the somewhat monolithic nature of the research community contributed to the drop in publications. However, if a system is truly robust, that is, it is tractable in a substantial number of laboratories and holds broad appeal for the advance of significant topics, the loss of one laboratory, even such a major one, should not compromise the entire publication record of the system. Certainly the topics of interest which can be approached in this organism have not disappeared or been solved completely in other systems. Given the potential of the system to make exciting contributions to developmental biology, why is the recent literature so devoid of publications?

> "The ultimate aim is, of course, to obtain sterile cultures in chemically defined media. . .: the difficulties to be overcome before we reach this goal are great, due to the length of the reproductive life cycle, . . ." ". . . in order to explore fully the possibilities given by *Acetabularia,* the obtention of mutants, . . . , is badly required. The long life cycle of the alga has so far prevented the initiation of studies of *Acetabularia* genetics: all efforts in order to speed up its development are valuable and might have important consequences." (pp. 274 and 289, respectively, in Brachet, 1970)

E. Solutions to Past Barriers to Use of the Organism

Three major barriers have prevented others from easily adopting the system despite its obvious potential and appeal. These are issues that must be resolved if this organism is going to have any long-term utility to the research community. While these may not be the only barriers, they are ones of paramount importance.

The first major barrier to the widespread use of *A. acetabulum* has been that the duration of the life cycle in the laboratory has been too long to make experimentation practicable. The life cycle in the wild is 2–3 years (Puiseux-Dao, 1962). In 1970, Shephard gave a detailed recording of the duration of each portion of the life cycle in the laboratory which totaled nearly a year (244 days; Fig. 5). By 1992, the duration of the life cycle had been reduced to ~6.5 months (197 days; Fig. 5 redrawn from Nishimura and Mandoli, 1992b). Although in 1997 a 6-month duration of the life cycle remains standard in many laboratories (VanDen Driessche *et al.,* 1997), a life cycle for heterogeneous, wild-type strains can take just 10–12 weeks (94 days; Fig. 5). Previously, the sheer duration of the life cycle hampered many types of developmental experiments and precluded genetic analysis of the organism (Brachet, 1970). Clearly, the duration of the life cycle is far less problematic if the experiments use only the vegetative phase. Also, many of the culture problems described here are far less important if the complete life cycle

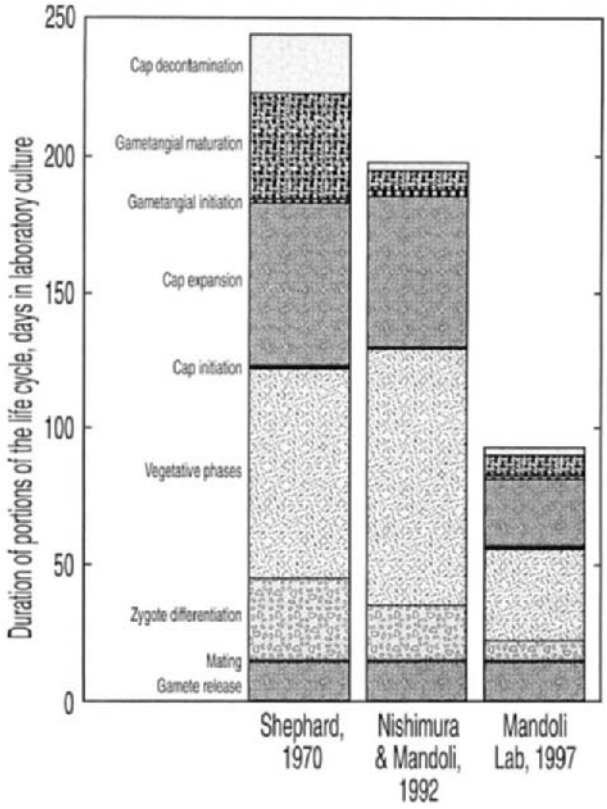

FIG. 5 Comparative durations of the life cycle of *A. acetabulum* in the laboratory documented in three publications in 1970, 1992, and in 1998 (this publication). Names of each portion of the life cycle are given to the left of the figure in register with the left column. Each portion of the life cycle has a distinctive shading common to all columns. The duration of each publication of the life cycle is indicated by its height and the total amount of the life cycle in each portion is represented by the height of each column.

is needed only to propagate stock cultures. However, when the goal is genetics or the study of late developmental events, the duration of the life cycle becomes prohibitive to the use of *A. acetabulum* as a model system.

A second major barrier to the widespread of *A. acetabulum* has been that traditional culture of the vegetative phase was labor intensive and all the progeny could not be recovered from reproductive cultures. Traditional culture demanded that the seawater medium be replaced every 2 weeks (Keck, 1964; Lateur, 1963; Schweiger *et al.,* 1977). When uncalcified in the

laboratory, these organisms were easily wounded (Fester *et al.*, 1993) and so the frequent changes of the seawater added recovery time to traditional culture. One alternative culture method was a flowthrough seawater system in which single, vegetative individuals are grown in separate tubes (Schweiger *et al.*, 1977). This flowthrough seawater system enabled very healthy algae to complete the life cycle and produced robust gametangia and fine progeny. However, the labor-intensive nature of either of these culture methods makes the growth of even a few hundred plants for classroom use tedious and renders genetics too expensive to be feasible. For example, determination of a segregation ratio ideally needs hundreds of individuals for a single Mendelian trait, and linkage group analysis requires thousands of individuals to make one confident of the result. Genetic studies also demand recovery and growth of all the progeny of specific, single-pair matings, so methods for efficient recovery and culture of progeny are essential. The rigors of the current funding climate and the pace of science in the 21st century will probably only serve to exacerbate the drawbacks of the long duration of the life cycle and the expense of culture of the organism; faster and cheaper is better.

The third major barrier has been that the culture methods precluded easy, intermittent use of *A. acetabulum:* a fair amount of trial and error was required to get cultures started, and then culture had to be continuous (i.e., no portion of the life cycle can be stored frozen), which has meant that the level of commitment to the system had to very high. The difficulties in starting cultures may have arisen because the physiological needs of the organism were unknown and because the delicate and/or critical steps in culture had not been clearly flagged. Some of the traditional culture methods require specialized equipment (Keck, 1964), are good only for portions of the life cycle (Lateur, 1963), and require proximity to a fresh, clean supply of running seawater (Schweiger *et al.*, 1977), and this has limited the number of labs that would even embark on culture of these species. In the last few years, culture has been streamlined, reliable conditions for long-term storage of the haplophase (gametangia) have been determined, and many of the problems in growing *A. acetabulum* have been ameliorated, so the organism is far more accessible to biologists with diverse needs.

Now, *A. acetabulum* are easy to maintain: we grow millions of plants at a time and have bred thousands of lineages. Using our current culture methods (Appendix: Table II), undergraduates with little or no bench experience grow the alga easily and generate publishable experiments on their own. However, the initial establishment of healthy cultures in this laboratory was not easy or rapid. Clearly, many of the problems encountered were related to our goal of performing genetic analyses. However,

we were forced to develop our own culture methods (Table II), even to establish the most routine culture because we could not get the algae to grow and reproduce as readily as we wished to. While we may have been "reinventing the wheel" from the perspective of those who could already grow the organism well, we found that the literature was not particularly helpful in guiding us as to what we were doing wrong in the basic culture of the organism, or, at times, even in helping us determine where the problems lay. Unfortunately, ours was not a unique experience: both the Algal Culture Collection of the University of Washington and the Algal Stock Center at the University of Texas have stopped culturing the species and no longer carry stocks in any stage of the life cycle. As we have moved into the position of helping others get *A. acetabulum* culture started in their laboratories, we are revisiting some of these same troublesome, albeit prosaic, issues through their eyes.

F. What Does the System Need Now?

What follows is a hard-nosed look at which technical limitations and which areas of ignorance of the biology have hampered past use of the organism and what remains to be resolved to improve culture and access to the genetics of this model system. To provide a logical framework for this discussion, the issues have been raised in the order in which they occur in development. I have tried to avoid maligning the literature or those who have previously tackled these issues. I intend no slight to others in addressing these issues and make every attempt to critique our own efforts equally hard as we do the work of others because, in the long run, this approach will produce the best science. Increased access to the system requires a frank appraisal of what would enable people to grow *A. acetabulum* for the greatest variety of purposes ranging from ongoing, pure research to periodic classroom use. In sum, one goal of this review is to facilitate access to *A. acetabulum* as a robust vehicle for research and teaching.

The following sections each have roughly the same format so that both the novice to the system and the more experienced researcher looking for help about the portions of development or culture with which they are encountering problems can most quickly find the information that they need. Salient features of each portion of the life cycle are described cursorily and the reader is referred elsewhere for additional details about the biology of the organism. In particular, this section highlights areas in which a lack of biological knowledge is hindering resolution of a culture problem or application of an important technique. Finally, remaining problems in culture which are particular to each portion of the life cycle are described and our best and most current solutions to them are proffered (see Table II).

TABLE II

Flow Chart of Culture for *Acetabularia acetabulum*

Desired event	Time needed	Optimal culture density	If healthy, you will see . . .	If unhealthy, you will see . . .	Most common cause of trouble and solution
Gamete release	1 min	1 gametangial pair per 200-μL mating matrix works well for single-pair crosses and for mass matings (see "Mating")	Empty gametangia with open or free opercula Gametes active and motile in mating matrix	**Dead gametangia:** Plasmolysed or white gametangia **Asynchronous:** Poor or sporadic release after 2 weeks	Start with new gametangia Pellet 5 min, 3k rpm; grow zygotes from supernatant, mate gametangia from pellet OR Incubate gametangia 1–2 months, 15°C in darkness; mate again
Mating	4–21 days	**Single-pair cross:** Use 2 gametangia **Mass mating:** Use anywhere from 3 to 10^8 gametangia	In a successful single-pair cross, *both* gametangia will empty, i.e., clear In a successful mass mate, a green meniscus will form at the surface of the seawater	**No mating:** Gametes can be trapped inside gametangia even when the operculum has opened or they can be dead (white)	Low K^+ in mating matrix; make new medium and remate gametangia OR Inoculate anyway if that cross is needed; there may be zygotes there
Zygote differentiation (initiation siphonous growth)	6–7 days	$\geq 10^4$ spherical zygotes/ml	Spherical zygotes become tube-like; ends of tube look similar	**Few zygotes** **Few siphonous juveniles**	Bad mating; mate again Bad genotype; start with new gametangia OR Culture too old, too dilute, or too crowded; adjust culture or start a fresh mating

Juvenile phase	15 days	10^2 early juveniles/ml	Stalk length increases slowly (0.2 mm per day) to ~10 mm A total of 5–6 whorls of 1–8 hairs in each whorl; whorl segments contain few if any chloroplasts Rhizoids grow rapidly, at 0.04 mm/day	**Few juveniles**	Too dilute; concentrate zygotes or start new zygote cultures
				Cytoplasm retracted: Cytoplasm shrinks in volume but rhizoid remains green as if cytoplasm is only in base of alga	Too crowded; dilute and incubate again OR Too old; inoculate zygotes again
Adult phase	20–35 days	1 late juvenile or early adult per 1.8 to 2.5 ml OR 18 to 25 late juveniles or early adults per 45 ml	Stalk length increases rapidly (0.6 mm per day) to a total height of 20–35 mm when a cap initiates A total of 10–15 whorls of 10–15 hairs in each whorl; most whorl segments contain chloroplasts Rhizoid grows slowly, 0.02 mm per day	**Cytoplasm mottled:** Cytoplasmic streaming uneven, "knots" of cytoplasm in stalk	Too much handling, i.e., daily; change medium and leave cultures alone
				Stalk tips abnormal: Stalk tips curled or bulbous	Poor genotype; use a different mating
				Whorls poor: Whorls look decayed or absent	Culture contaminated; sterilize and start over with axenic gametangia OR Too crowded in adult; dilute for caps
				Medium green: Seawater turns green or cloudy; bottom of dish green or has green spots	Contaminated with microalga; STERILIZE EVERYTHING and start fresh; cleaning dishes or organisms does not work well
				Mass death: All adults turn white and die overnight	Cause unknown; STERILIZE EVERYTHING if pathogen suspected and use fresh mating

(*continued*)

TABLE II (*continued*)

Desired event	Time needed	Optimal culture density	If healthy, you will see . . .	If unhealthy, you will see . . .	Most common cause of trouble and solution
Cap initiation	1 day	Same as for adult growth; change of medium is not needed	Cap initial is knob shaped	**No caps initiate**	Too crowded in adult phase; dilute adults or start with younger culture OR Not wild type; start with a new zygote stock
				Cap aborts: Cytoplasm absent in old cap and new stalk grows	Causes unknown; let intact algae or amputatees form a 2nd cap in new medium
Cap expansion, nuclei and cytoplasm transported to cap	23–30 days	Same as for adult growth; change of medium is not needed	Cap round and symmetrical with a flat, saddle, concave, or convex shape Cap diameter increases 0.2–0.4 mm per day to a final diameter of 5.6–10 mm Nuclei and cytoplasm visibly streaming into cap at stalk junction	**Caps oddly shaped:** Most caps small, asymmetrical, or disorganized	Too crowded in adult phase; dilute adults or start with younger culture OR Photon flux density low; adjust light level
				Cytoplasm in stalk: Stalk retains cytoplasm *post*-nuclear transport	Too crowded in adult phase; dilute adults

Gametangial initiation	1 day	Same as for adult growth; change of medium is not needed	Gametangia symmetrical, round, or oval in shape Stalk junction clear and sealed off from cap Gametangia light green and all walls clear	**Gametangia form in stalk** **Cytoplasm clumped:** No gametangia in the cap, only stringy or clumped cytoplasm	Too crowded in adult phase; dilute adults Metal toxicity; make fresh medium OR Cause unknown; use new adults
Gametangial maturation	3–7 days	Incubate in 4 ml per cap of fresh medium Higher light level *may* speed maturation	All gametophores filled evenly with gametangia Gametangia green, packed at outer rim of cap	**Gametangia asymmetrical or oddly shaped**	Too crowded in adult phase; gametangia usually mate normally OR Poor genotype; use a different mating
Cap decontamination	3 days		Cap wall birefringent and free of debris Ends of gametophores and stalk partially calcified Stalk clear, cap may be loosely attached if at all Gametangia dark green, full of cytoplasm and nuclei	**Cap contaminated:** Microbe colonies evident even after incubation on rich broth [**Cap falls apart:** *Healthy,* older caps become flaky or rupture as the cap wall loses integrity] **Gametangia dead:** Most (>75% in a cap) gametangia plasmolysed or white	Decontaminate again; if this fails, isolate gametangia and reincubate in antibiotics Collect gametangia and mate; if a controlled cross is desired and genotype is now unsure, start over Silver protein step too long or too strong; start with fresh gametangia-bearing caps OR Poor genotypes; use a fresh zygote stock

II. The Haplophase

A. Gametangia

In healthy heterogeneous wild-type cultures, fully formed gametangia are spherical, ovoid, or, rarely, tricornered in shape and vary in color from light green to dark green. Gametangia averaged 101.87 ± 25.89 μm in diameter (Nishimura and Mandoli, 1992a). Large, oddly shaped gametangia, e.g., tube-like or pear-shaped, occur under unhealthy culture conditions or in strains with genetic defects (B. E. Hunt and D. F. Mandoli, unpublished observations). Gametangia house maturing gametes and may serve as the resting structure for the species in the wild.

1. Uses of Gametangia

The hardy, thick wall makes the gametangia ideal for chemical treatments such as removal of other organisms from cultures, or "decontamination" (Hunt and Mandoli, 1992; Mandoli *et al.,* 1995), and allows long-term storage of the germplasm of the species (Hunt and Mandoli, 1998). Since each gametangium contains an average of 1561 nuclei (Mandoli and Larsen, 1993) with a mean of 4.4×10^3 gametangia per alga (Nishimura and Mandoli, 1992a), they are also the best source of DNA for the species (Table I). Because the organism is monoecious but an individual gametangium always contains gametes of just one mating type or the other (Green, 1973; Koop, 1975b; Mandoli and Larsen, 1993), gametangia are very useful for the self- and out-crosses essential to genetic studies.

A. acetabulum grows best when microorganisms such as bacteria, yeast, and other algal species with faster generation times are absent. Because these other organisms have shorter generation times than *A. acetabulum* and impede access to or compete very effectively with it for nutrients and/or light, developing effective means to rid cultures of other organisms has proved a crucial step in improving culture of *A. acetabulum.* What the nonphotosynthetic epiphytes were living on is not known. There are at least five published methods of removing other organisms from the caps or gametangia of *A. acetabulum* (Hunt and Mandoli, 1992). We use a simple, rapid method for removing organisms from the cap exterior and the enclosed gametangia which is >90% effective, works well on single caps or in batches of several hundreds (Hunt and Mandoli, 1992), and has proved to be effective for all the wild-type, inbred strains, and other species of *Acetabularia* that we have tested so far (B. E. Hunt and D. F. Mandoli, unpublished observations). With this method, if an intact cap was not rendered axenic (from the Greek, meaning "without guests") by treatment

the first time, the cap can be retreated as a unit, or the gametangia can be isolated from the cap and treated with the same combination of antibiotics (Hunt and Mandoli, 1992) or a slightly modified one (Mandoli *et al.,* 1995) with no ill effects. It is not necessary to keep cultures absolutely axenic for many types of experiments such as genetics and routine culture of algae for classroom use. However, if the culture is not rendered axenic for a few generations, the competing microorganisms will bloom, overtake, and can even kill the *A. acetabulum.* Transferring the desired *Acetabularia* to fresh medium does not solve the problem because many of the contaminating microbes are good epiphytes and are simply transferred to the new dish attached to the outside of the *Acetabularia.* Since morphologically normal, healthy *A. acetabulum* cultures have been grown axenically, or nearly axenically, in our lab for over seven generations, we concur with Green (1973) that concerns of previous authors (Puiseux-Dao, 1962) about the ability of the alga to complete the life cycle when axenic can probably be put aside.

The ability to store gametangia for ≥1 year (Hunt and Mandoli, 1998) obviates continuous culture, permits the preservation of germplasm, and provides a ready source of gametes for genetic crosses. None of the standard means for storing other organisms, such as freezing in 7% DMSO or 50% glycerol, or any variations of these (e.g., varying the rates of freezing or thawing, etc.), worked well (Hunt and Mandoli, 1998). However, 89% of wild-type gametangia released after 15 months at 15°C in darkness in seawater (Hunt and Mandoli, 1998). Although reliable long-term storage of gametangia is a boon to genetic analysis, relief from continuous culture is perhaps of greater value because it makes culture far less labor intensive and, for the first time, makes the system attractive to the periodic or casual user.

There are significant advantages to storing gametangia rather than zygotes. Zygote stocks are more labor intensive to care for: zygotes need to be exposed to photosynthetically active light for 3 days on a monthly basis with a change of seawater every 3–6 months (Berger and Kaever, 1992), so periodic users, such as the biology teacher, may not be willing or able to care for zygote stocks. Less space is required to store gametangia than is required for zygotes because zygotes die when stored under crowded conditions but gametangia do not. An added bonus to cold storage of gametangia is that it tends to synchronize gamete release (Hunt and Mandoli, 1998; Mandoli and Larsen, 1993) and so increases the probability of successful single gametangial crosses to close to 50% (Hunt and Mandoli, 1998), the theoretical maximum given two mating types.

2. Challenges with Gametangia

A minor technical issue is that only properly matured gametangia survive the decontamination process that we use (Hunt and Mandoli, 1992). Gam-

etangial maturation depends both on the volume of the seawater per individual alga and on the duration of the incubation for maturation (Geil and Mandoli, 1998b; Hunt and Mandoli, 1992). When gametangia are not matured properly in excess seawater, they die during the decontamination process (Geil and Mandoli, 1998b; Hunt and Mandoli, 1992), perhaps because they are still too permeable to the exterior environs. Most strains do very well when their gametangia-bearing caps are incubated for 8 days in 4 ml of medium per cap so this is a good place to start in handling an unknown or new lineage. The exact volumes and times required vary slightly from ecotype to ecotype (Hunt and Mandoli, 1992) and on the growth conditions of the organism during the vegetative phase but it is trivial to add more seawater per individual and increase the duration of the incubation for a few days. It is possible that the seawater might be inadequate for gametangial maturation but death of immature gametangia during decontamination cannot be ascribed solely to poor physiology until the contribution of lethal genes can be assessed.

After decontamination of intact caps, gametangial viability and biological variation in gametangial maturation are two areas in which an incomplete knowledge of biological issues impacts the handling of the alga in culture. First, more of an irritation for some types of experiments than a real problem, is that gametangia within an individual cap are not all viable. In heterogeneous wild-type plant populations, 75–80% of all gametangia are viable. Consistent with the presence of lethal alleles, while making inbred strains, gametangial viability in two ecotypes has been as low as 10% for the first three to four generations (B. E. Hunt and D. F. Mandoli, unpublished observations). Later inbred generations showed gametangial viability better than that of their wild-type parents. These data suggest that higher gametangial viability may be at hand and might be attributed to genetic rather than physiological causes in at least two heterogeneous, wild-type ecotypes. Second, gametangia isolated from matured, axenic caps can take a long and variable time to release gametes. This poses a nuisance because asynchronous gamete release can introduce significant delays in experiments, asynchrony in the outgrowth of progeny, and can stall completion of the life cycle. In our hands, synchrony of gamete release is unaffected by the conditions under which intact gametangial-bearing caps are matured prior to decontamination. For example, variability in the timing of gamete release is not altered either by increasing the volume of the seawater in which the caps are matured or by extending the duration of the incubation (B. E. Hunt and D. F. Mandoli, unpublished observations). [A related topic, induction of gamete release, is discussed below.] Koop (1975a) postulated that there was a requisite dormant period in gametangial maturation because gametangia released synchronously and to the maximum percentage only when they were 17 weeks old. However, gamete release can occur as rapidly as 4 days after isolation of gametangia from the caps (Hunt and Mandoli, 1992). Two

significant differences between these reports were that Hunt and Mandoli (1992) used caps that had been exposed to a protease during decontamination prior to the induction of gamete release and the seawater mixture in which the gametes were induced to release differed. Greater synchrony of gamete release was obtained if axenic caps were incubated for 1 month at 15°C in the dark (Mandoli and Larsen, 1993), but this increased the effective duration of the life cycle in the laboratory. Resolution of whether gametangial dormancy exists might eliminate the nuisance inherent in the variable maturation time and, if dormancy is real, might augment long-term storage of gametangia.

B. Gametes

An average gametangium makes 1561 ± 207 gametes (Mandoli and Larsen, 1993). Unlike *Chlamydomonas,* the mating types have no distinguishing ultrastructural features that have been identified, i.e., they may be completely macroscopically isogamous (Crawley, 1966, 1970; Hämmerling, 1932; Puiseux-Dao, 1962). Gametes are about 5 μm in diameter (see Fig. 1 in Crawley, 1966), are bounded only by a plasmamembrane, i.e., they lack a wall, and contain one horseshoe-shaped chloroplast each (Crawley, 1966, 1970; Herth *et al.,* 1981). Gametes are biflagellate with a cruciate flagellar root (Herth *et al.,* 1981). The cruciate roots of the flagella are intimately associated with a bilayered eyespot that is about 3.5 μm long (see Fig. 3 in Crawley, 1996) and about 1.5 μm^2 in area (Melkonian and Robenek, 1984). Gametes will swim toward light reportedly over distances of 10 cm (Keck, 1964).

Release of gametes occurs through a lid or "operculum" in the wall of each gametangium. Healthy gamete release is explosive and rapid, finishing within a minute or two (Koop, 1975a; Mandoli and Larsen, 1993). Gametes swim rapidly free of the gametangium from which they emerged. Good gamete release is often presaged by movements of gametes inside the intact gametangium prior to opening of the lid, or operculum. If gametes fail to escape the gametangial wall, release is considered "abortive" (Mandoli and Larsen, 1993). In contrast, poor gamete release is marked by incomplete opening of the operculum and partial exodus of the gametes from the gametangium. During poor release, gametes sometimes look as if they are attached to the gametangial vesicle, unable to wiggle free of the outer surface of the vesicle membrane. Gametes can also activate but then fail to release from the gametangium.

1. Uses of Gametes

Stable haploid cultures of *A. acetabulum* have been reported (Dübel *et al.,* 1985) but the means to generate and to stabilize the haplophase have not

been developed. The DNA content of these algae was half that of diploid organisms as assayed by microspectrophotometry (Dübel *et al.*, 1985; Spring *et al.*, 1978). In contrast to diploid zygotes, these haploids never became siphonous but remain as unicellular, misshapen blobs (Dübel *et al.*, 1985). Gametes that do not mate would be the logical progenitors of haploids but this has not been demonstrated. During our inbreeding of homogeneous wild-type lineages, about 5.5% of the progeny of self-crosses in the first five generations (out of 933 self crosses; B. E. Hunt and D. F. Mandoli, unpublished observations) produce individuals that never become siphonous, "nubs" (Mandoli, 1996). These individuals either could be zygotes incapable of differentiation or of siphonous growth or could be stable haploids. Clearly, development of a method that would enable stable haploids to be generated at will would be an important tool for genetic analysis.

A *priori* gametes seem like a poor choice for storing germplasm: gametes lack a wall, have just one chloroplast, and have a high rate of activity until they die or lose their flagella after mating. Whether the half-life of gametes depends on the presence of the opposite mating type has not been gauged. In the presence of the opposite mating type, most gametes remain unmated only for about 24 h with a half-life of 14.5 days (Mandoli and Larsen, 1993). However, on occasion we have seen gametes swimming in zygote stocks placed at 15°C for storage, months after the majority of the gametes have released (T. Larsen and D. F. Mandoli, unpublished observations). This result suggests that it may be possible to store the haploid form by reducing the temperature soon after release. Whether the gametes that survived these low temperatures were capable of subsequently mating is untested.

2. Challenges with Gametes

Synchrony of gamete release is variable and therefore causes major problems in subsequent culture of the alga because the resulting zygotes are developmentally asynchronous. Within a single mating, gamete release can take from 4 to 21 days and can occur all in 1 day or spread out over weeks. From mating to mating the timing of release and the duration of the burst of release is unpredictable even when the same pool of gametangia is used. The effect of this asynchrony in gamete release to subsequent culture is that when the zygotes resulting from the earliest matings have begun juvenile or even adult growth, the zygotes from the latest gametes released are just beginning to differentiate. This forces cultures to be manually "thinned" to obtain cohorts of individuals at the correct population density which are also developmentally synchronous. Thinning of cultures is time consuming, labor intensive, and risks contamination of cultures. Although a pragmatic approach to creating developmentally synchronous zygotes stocks has been developed (Cooper and Mandoli, 1998), the relative contributions of physio-

logical and genetical factors to this problem have not been resolved. In sum, many of the major problems in the culture of *A. acetabulum* can be attributed to the inability to induce consistent, synchronous gamete release.

C. Mating

Matings can be a self-cross between gametangia from a single cap, or an out-cross between gametangia from two or more different caps because each individual plant contains gametangia of the mating types (Fig. 1). Mating occurs within about 1 h of gamete release. The orientation of the gametes to each other, the swimming of the quadriflagellate pair, the kinetics of gamete, and nuclear fusion have been cursorily described (Hämmerling, 1934a). When a pair is firmly made, the resulting quadriflagellate, binucleate zygote swims away from light (Hämmerling, 1934a; Keck, 1964; Shephard, 1970). This photobehavior presumably functions to place the diplophase on the bottom of shallow lagoons in which the adults will grow and develop. A successful mating can be easily recognized because the gametes will form a green halo at the meniscus of the mating medium and because zygotes are produced. Conversely, a failed mating can be recognized by a halo of dead, white gametes around the gametangia, a lack of zygotes, and empty gametangial shells and opercula in a liquid mating matrix or on a solidified mating matrix (not shown).

1. Uses of Mating

Efficient mating is, of course, essential to genetic studies. There are two categories of mating: a "mass mating" in which more than two gametangia are mated and a "single-pair mating" in which just two gametangia are mated. Note that these categories are applicable both to self-crosses and to out-crosses. Mass matings are used for propagation of ecotypes or strains and, based on the size of the genome, will preserve the genetic diversity of the organism if $\geq$200 individuals are used per mating (D. F. Mandoli and D. Schemske, unpublished calculation). Both mass and single-pair matings yield close to or more than the theoretical number of zygotes expected (Mandoli and Larsen, 1993; B. E. Hunt and D. F. Mandoli, unpublished observations). Single-pair crosses, regardless of whether they are self- or out-crosses, at best have a 50% probability of producing progeny (Green, 1973; Mandoli and Larsen, 1993) because the mating type of the gametes inside a gametangium is not obvious from the gametangial exterior and gametangia are not physically arranged within the cap according to sex (Geil and Mandoli, 1998a). In addition, the probability of obtaining a

successful mated pair from just two gametangia can be compromised by the lack of synchrony of gamete release.

2. Challenges with Mating

Fortunately, mating contributes no problems to culture of *A. acetabulum.* Although there remains a finite probability that there are more than two mating types (Geil and Mandoli, 1998a), this does not impede culture or genetic studies at present. All of the problems that we have encountered with mating center around the inability to induce synchronous gamete release and the inability to form healthy and mature gametangia reliably during the reproductive portion of the life cycle. Some of the matings which fail to yield zygotes may be attributable to mating defects present in the heterogeneous, wild-type strains but this remains conjectural.

III. The Diplophase

A. Zygotes

Zygotes are spherical in shape and are enclosed in a wall. During or immediately after mating, nascent zygotes will attach to each other, to the walls of container in which they are mated, or to any agar particles in the mating matrix. Epifluorescence microscopy of glutaraldehyde-fixed zygotes sampled at several times after mating suggests that there is uniparental inheritance of the chloroplast genome in *A. calyculus* (Kuroiwa and Shihira-Ishikawa, 1985). Consistent with this, two isozymes of a Ca^{2+}-dependent nuclease which are thought to be specific for the destruction of the plastid genome and are donated by the − mating type in *Chlamydomonas* were also found in *A. calyculus* (Nakamura *et al.,* 1987). Direct evidence that microscopy and nuclease activity were strictly correlated remains lacking, as does genetic evidence for uniparental inheritance of chloroplasts in any *Acetabularia* species. (Note, however, if this nuclease was shown to be specific for just one mating type in *Acetabularia,* and if it was always the chloroplast donated from the same mating type that was destroyed, this may be a candidate for a sex-specific trait and might provide a way to clearly distinguish the mating types.) Just after mating, zygotes of *A. acetabulum* are roughly 10 μm in diameter (Crawley, 1966, 1970). Zygotes first become larger in diameter and, by about 7 days after inoculation into fresh seawater, differentiate a rounded outgrowth at one end (Cooper and Mandoli, 1998). Whether it is the apical or basal pole that differentiates first is not clear.

1. Uses for Zygotes

Zygotes are useful for long-term storage of genotypes of *A. acetabulum.* Zygote stocks are viable for 1–2 years when stored at 15°C in the dark with periodic illumination (1–3 days per 6 months; Berger and Kaever, 1992; Schweiger *et al.,* 1977). Zygote stocks also are useful as "nursery" cultures for later phases of the life cycle (Cooper and Mandoli, 1998; Lateur, 1963; Zeller and Mandoli, 1993). Zygotes differentiated readily, 80% within 6–7 days, but only when the population density was high, $\geq 10^4$ per milliliter (Cooper and Mandoli, 1998; Zeller and Mandoli, 1993). Zygote differentiation had a broad temperature optimum between 18.6 and 23.5°C and was optimal when there were $>5 \times 10^3$ individuals per milliliter at a pH of 7.96, and a potassium concentration between 1 and 10 m*M,* but was independent of the photon flux density of cool white light (Cooper and Mandoli, 1998). Still problematic is the fact that the high population densities needed to differentiate zygotes are directly at odds with their viability in long-term storage: if they are dense enough to differentiate easily, they are too dense to store well (Cooper and Mandoli, 1998).

Zygotes are also valuable to algal culture because their size allows them to be pipetted and serially diluted easily. Algae that are unattached to any others are desirable because they grow faster and more synchronously through the rest of vegetative and reproductive growth than do clumps of organisms (Schweiger *et al.,* 1977) and because they are important for genetic studies. In the past, the tendency of zygotes to stick to each other and to solid objects in the mating environment posed severe problems to culture. It is not known what zygotes produce which allows them to attach to substrata or to each other. The tendency of zygotes to stick to each other made isolation of individual algae very difficult. Although zygotes can be scraped off most smooth solid surfaces, they come off in sheets and then are impossible to separate from each other. If these sheets of algae are allowed to grow, the organisms do become siphonous but intact reproductive plants cannot be pulled free without damaging the rhizoids. Because the stalk continues to differentiate in the absence of the nucleus, whether or not the entire rhizoid came along with the reproductive alga is not apparent until weeks or months later when the expanded cap either does or does not fill with gametangia. The agar matrix in which we mate gametangia apparently prevents most of the zygotes from sticking to each other or to the culture container if the mating is rocked slightly during gamete release. If the gametangial slurry is not mixed during mating, zygotes clump wherever it is darkest, i.e., under the writing on the tube and near the lid. Mating under these conditions provides suspensions of algae which are ≥90% single zygotes unattached to their siblings. The primary disadvantage of this matrix is that zygotes cannot be freed from it until they begin siphonous growth.

Centrifugation pellets both the agar and the zygotes, especially since zygotes tend to attach to the agar particles in the matrix. Although the matrix is a low-melting-temperature agar, the temperature at which the agar melts is high enough to kill the zygotes (Cooper and Mandoli, 1998; Mandoli and Larsen, 1993). Enzymes which digest agar (i.e., agarase) also require an elevated temperature that kills zygotes. Although the agar matrix is not perfect, its use has obviated the problems arising from the tendency of zygotes to stick to solid surfaces and to each other immediately after mating.

2. Challenges with Zygotes

One aspect of zygote biology that complicates the mass matings required for culture is that the differentiation of zygotes is asynchronous. Recall that gamete release is also asynchronous. It is hard to say whether the asynchrony of zygote differentiation is a separate problem or merely a consequence of the asynchrony in gamete release. We have not been able to isolate zygotes at homogeneous stages in development using physical means, e.g., Percoll gradients (B. E. Hunt and D. F. Mandoli, unpublished observations). Fortunately, one can obtain mass-mated stocks composed of zygotes which are fairly synchronous in development by removing the subpopulation of gametangia that has not released their gametes (3000 rpm for 5 min; Cooper and Mandoli, 1998). Gametangia that are still full of gametes will pellet whereas those that have released will not pellet. The supernatant serves as a fairly developmentally homogeneous zygote stock and the pelleted gametangia can be resuspended in fresh mating matrix to obtain more zygotes even months after the original mating (Cooper and Mandoli, 1998). Single-pair matings tend to be far more synchronous in zygote differentiation presumably because the gametangia had to release roughly at the same time to obtain any zygotes at all. However, sibling zygotes can still be asynchronous. Thus, the complete solution to the problem of asynchronous zygote differentiation, i.e., the relative contributions of asynchrony in the time of mating and competition between zygotes for nutrients, light, etc., is not yet at hand.

We know nothing about the factors that control zygote differentiation and this causes a major problem in the culture of progeny of single-pair matings, independent of whether the mating was a self- or an out-cross. Only 25% of the zygotes differentiated after about 10 days when the zygote concentration was $<3 \times 10^2$ zygotes per milliliter (Cooper and Mandoli, 1998). Obtaining a high population density is not usually an issue for propagation of robust lineages which have been mass mated although it may take a few tries to obtain the desired density. However, obtaining a high population density of zygotes is a significant problem for growing out the progeny of single-pair matings as the following calculations illustrate. There will be no problems with zygote differentiation if 100% of the zygotes

resulting from pairing two gametangia are viable and the matings are done at the optimal volume, 0.2 ml (Mandoli and Larsen, 1993), because then the population density will be high enough. Given that on average a gametangium releases 1561 ± 207 gametes (Mandoli and Larsen, 1993), there will be 1.6×10^3 zygotes per 200 μl or about 7×10^4 zygotes per milliliter. However, these yields of zygotes per gametangial pair are theoretical and do not take into account several factors, each of which can reduce the number of zygotes formed. First, the number of viable progeny will vary as a function of the number of gametes per gametangium (that in one wild-type strain ranged from 167 to 6440 gametes per gametangium; Mandoli and Larsen, 1993). Second, spatial and temporal constraints on mating can also impact the number of zygotes made by a gametangial pair. Gametes have a finite half-life (14.5 days; Mandoli and Larsen, 1993), may have limits as to how far they can swim prior to mating, and need time to find each other. So, for example, if two gametangia in a pair release their gametes 10 days apart, the mating may succeed but will yield few zygotes. Finally, the number of recessive lethal genes that become homozygous in a particular mating will impact the number of viable zygotes that result. In practice, the probability of obtaining one good zygote stock, i.e., one from which any wild-type heterogeneous adults could be grown, was 4.2% out a total of 497 single-pair matings of fresh, wild-type gametangia (Hunt and Mandoli, 1998). Note that the contribution of each factor to this overall probability was not established. Setting up each single-pair mating took only 1 min—a relatively trivial time investment. Even if synchronizing gamete release improved mating success to 50%, the theoretical maximum given only 2 mating types made in equal numbers, our lack of understanding what induces zygote differentiation would *still* be an impediment because zygotes at a low population density take a long time to differentiate. Clearly, understanding the biology of zygotes would be desirable because it would lead to reliable means of inducing differentiation independent of population density (e.g., growth factor(s), conditioned medium, physiological factors, etc.).

A potential use of zygotes is for genetic selections at an early stage of development when the algae are small and capable of being pipetted like a microbial cell culture. Theoretically, even a single-pair cross will yield ≥1000 zygotes, which is more than enough to rescue a trait of interest even if there is a lethal recessive segregating in that cross. Genetic selection of zygotes looks feasible and promising (Geil and Mandoli, 1998b).

B. Vegetative, Siphonous Growth

Vegetative growth commences when the zygote differentiates a clear polarity and ends with differentiation of a cap initial at the apex of the alga.

The term "siphonous" is from the Greek meaning "tube or straw-like" and describes both the growth habit and the final shape of the vegetative stalk of the organism. Each siphonous, vegetative alga has three main anatomical features: a basal rhizoid, an apical tip, and sets of lateral extensions or hairs which are arranged in whorls and spaced fairly evenly along the stalk (Fig. 1).

Healthy individuals are light to deep green in color depending on the ecotype or lineage, and on the seawater we use (Hunt and Mandoli, 1996); wild-type individuals made a cap initial 35 days after zygote differentiation when they reached 2–3 cm in length (Nguyen and Mandoli, 1998). Healthy individuals have a single, pointed apex which grows quickly, have a rhizoid composed of $\geq$10 fingers with blunt tips, and retain most of their whorls during vegetative growth. In contrast, unhealthy algae are gray–green or brown–orange in color and abort their caps repeatedly or fail to make a cap at all for up to 1.5 years in culture. During this prolonged vegetative growth period, unhealthy individuals reach lengths of 8–10 cm. Unhealthy individuals have a rounded or bulb-shaped tip (Goodwin *et al.,* 1983) and a relatively normal looking rhizoid but tend to be whorlless since they fail to retain their whorls or to initiate new ones at regular intervals. Unhealthy individuals result from poor physiological growth conditions such as overcrowding (Cooper and Mandoli, 1998; Nguyen and Mandoli, 1998; Zeller and Mandoli, 1993), poor nutrient status (Goodwin *et al.,* 1983; Hunt and Mandoli, 1996; Nguyen and Mandoli, 1998), or inadequate light quantity or quality (Beth, 1953; Clauss, 1970; Cooper and Mandoli, 1998; Kratz and Mandoli, 1998b) and can also result when they are grown in certain types of culture vessels (Table III). Another species, A. *calyculus,* has a similar suite of morphological variation when it is unhealthy (Shihira-Ishikawa *et al.,* 1985). The health and morphology of both *Acetabularia* species can be improved if their apices are amputated and if the amputated rhizoids then are placed under better physiological conditions (Berger and Kaever, 1992; Goodwin *et al.,* 1983; Shihira-Ishikawa *et al.,* 1985).

1. Uses of Vegetative Algae

A primary use of vegetative organisms has been for studies of developmental potential of regions of the alga after amputation (Fig. 3). Prior to amputation of a portion of the alga, a break or discontinuity in the cytoplasm is induced to prevent a loss of contents during the subsequent amputation. Individuals either can be "ligated," in which a slip knot in a silk thread is tightened around the stalk (Keck, 1964), or can be "pressure-wounded," in which the stalk is compressed perpendicular to the long axis of alga with a thin blunt tool (Fester *et al.,* 1993; Mandoli and Hunt, 1996). These cytoplasmic discontinuities can be made without breaching the wall and

cause a retraction of the vacuole and the cytoplasm away from the site of wounding (Fester *et al.,* 1993). This simple method is powerful because segments of the alga live for months independent of the presence or absence of the nucleus and are capable of significant growth and development (Hämmerling, 1963), because amputation separates important functional portions of the alga from each other (e.g., nucleus, apex, and cytoplasm), and because the healing process that is induced enables the pieces of algae to be grafted together.

A second important use of vegetative algae has been for construction and analysis of grafts (Fig. 3). Such graft chimeras are useful for studying the interactions between the rhizoid and the apex during subsequent developmental events in the life cycle of the alga. Although chimeras can mix and match grafts between two or more pieces of different individuals (Bonotto *et al.,* 1971), most frequently, grafts have joined the rhizoid of one individual to the apex of another or have joined the rhizoid of one individual to the rhizoid of another. Note that the rhizoid::apex graft joins the apical end of the rhizoid to the basal end of the apex, whereas the rhizoid::rhizoid graft joins the apical ends of the rhizoids to each other (Fig. 3). Typically, the graft partners are chosen to have either different genetic backgrounds or a different physiological status. For example, classic experiments used grafting to examine the contributions of the nucleus and the cytoplasm to events of interest. Hämmerling (1934b) used interspecific grafts to assess when the cytoplasm and when the nucleus governed the morphology of the cap made by the graft. Werz (1955) used interspecific grafts to examine the effects of nuclear dosage on specific features of cap morphology. Schweiger and colleagues (1964) used grafts to assess whether the apex or the rhizoid controlled the circadian rhythm of the resulting graft. Bonotto (1989) used intergeneric grafts to document graft incompatibility (death of one graft partner) between phylogenically divergent species. The availability of mutants of *A. acetabulum* have made mutant::wild type and mutant::mutant grafts feasible (Fig. 3; Mandoli and Hunt, 1996). While it is too early to tell how the biological and the genetic analyses of mutant graft chimeras will compare in the long term, it is clear that the potential of grafting as a bioassay has barely been tapped.

2. Challenges with Vegetative Algae

There were two major problems in the growth of vegetative adults. First, obtaining high numbers of healthy organisms that were fairly synchronous in development used to be problematic. However, we have recently found that the timing of dilution of nursery cultures determines how many of the young siphonous individuals in a population initiate the first whorl. For example, zygote cultures must be diluted when 80% of the population has

differentiated, about 6–7 days after the start of a nursery culture, or else zygotes either fail to initiate any whorls or die (Cooper and Mandoli, 1997). In addition, to obtain healthy, developmentally synchronous cultures, it is necessary to culture juveniles at a density intermediate to that of zygotes and adults (Table II). Recognizing the need to reduce population density at a particular time in development has greatly ameliorated this first problem in growing vegetative adults.

The second major problem in the growth of vegetative adults has been that populations of vegetative algae tend to exhibit "mass death," a phenomenon in which all the individuals in a culture vessel die and turn completely white literally overnight and without any warning signs. Although on average such dramatic death of entire populations affects just 1 culture vessel out of 4 to 5 in heterogenous wild-type lineages, it is an annoying phenomenon because it tends to occur frequently in some inoculations but not in others made from the same mating. For example, in an experiment, 3 out of 5 replicates of 1 treatment will die but only one 1 out of the 20 other experimental treatments will be affected. Of course, it is usually the critical datum or the control which experiences the most death. Mass death in this species has been clearly correlated with population densities that are too high (Zeller and Mandoli, 1993). However, sometimes even when the population density has been properly adjusted for that phase of vegetative growth, algal populations die anyway. One possible explanation of this paradox is that the timing of the adjustment of population density is the variable that determines whether or not mass death will occur at some later

TABLE III
Effect of Culture Vessel on Reproduction of *A. acetabulum*

	Mean maximum percentage			
	Ace seawater derivative		Ace-25	
	Culture flasks[a]	Square petri dishes[a]	Round petri dishes[b]	Square petri dishes[b]
Cap initiation	7 ± 3	85 ± 3	33	100
Gametangial formation	0	80 ± 1	8	100

[a] The baseline medium for these experiments was modified Müller's (Schweiger *et al.*, 1977) with the chelator for iron changed to EDDHA (1:1), and the H_2O quality, RbCl, CoCl, NaBr, and KI altered to the values in Ace-25 (Table I). A stable (i.e., unchanging) mean maximum percentage cap initiation was reached by Day 85 postinoculation. Density was 5 ml per alga.

[b] The baseline medium for these experiments was Ace-25 (see Appendix). A stable (i.e., unchanging) mean maximum percentage cap initiation was reached by Day 69 postinoculation. Density was 2.5 ml per alga.

time but this has been hard to prove (J. Cooper, K. Nguyen, and D. F. Mandoli, unpublished observations). Depletion of nutrients in the medium or a toxin produced by the alga when they are too crowded are both possible causes of this phenomenon. Alternately, such population crashes may be pathological in nature, reflecting the lytic phase of a marine virus. Interestingly, this phenomenon has only been seen in populations of heterogeneous, wild type but has never been observed in any of our robust inbred lineages (B. E. Hunt and D. F. Mandoli, unpublished observations). Mass death remains of concern in the culture of heterogeneous, wild-type *A. acetabulum.*

Adults are not easily stored for long periods of time. Arrest of growth of vegetative siphonous adults was possible at 15°C in the dark and maintained some algae in partial stasis for several years (Schweiger *et al.,* 1977). In our experience, however, both wild-type and putative mutants stored in this way sometimes developed in ways not seen under the normal growth conditions. For example, the mutant *kurkku* became approximately four times the normal height when stored under these conditions (B. E. Hunt and D. F. Mandoli, unpublished observations). In addition, once returned to normal growth conditions, both wild-type and mutant strains that have been stored often die. In sum, while the abnormal growth in these lower temperatures may be encouraging for development of methods for screening for cold-sensitive alleles, at present it is not attractive for long-term storage.

C. Reproductive Onset

Reproduction initiates with the differentiation of a doughnut-shaped structure at the apex of the alga. In our terminology, cap initiation is the first event in "cap formation" which encompasses all of the subsequent reproductive development, e.g., cap expansion and the nuclear events which occur later (Runft and Mandoli, 1996). Note that this definition differs from others which do not consider reproduction to begin until the haploid daughter nuclei are visible in the alga (Bonotto, 1988).

1. Uses of Reproductive Onset

Reproductive onset is a useful model for differentiation, because it is an explicit differentiation event which is parallel to flower initiation in vascular plants, and because it is probably light-regulated (Clauss, 1970). The earliest change that signals reproductive onset is the formation of a knob-shaped stalk apex (Kratz *et al.,* 1998). Whereas the vegetative apex alternates between a dome- and a cone-shaped apex, at cap initiation, the stalk apex clearly becomes rounded like the knob on a door. Although there may be

a slight swelling of the stalk apex prior to knob, this shape change was not always seen and when it was, it was not a sufficiently reliable indication that a cap would be formed. However, because 95% of the organisms with a knob-shaped apex subsequently completed cap formation, knob is the earliest reliable morphological evidence of reproductive onset (Kratz *et al.*, 1998). The photobiology of cap initiation is largely unknown.

2. Challenges with Reproductive Onset

In the past, there have been two problems in culture which have impeded cap initiation in *A. acetabulum:* the first major problem was that obtaining reproductive onset was very difficult without an enormous amount of labor-intensive algal culture. In traditional algal culture, the volume of seawater needed per individual was kept low but the seawater had to be replaced weekly or biweekly (Berger *et al.*, 1987; Schweiger *et al.*, 1977). This made culture labor and space intensive, as well as expensive. We calculated that 200 ml of seawater per individual was needed to obtain cap initiation (Zeller and Mandoli, 1993). Improvement of the nutrient environment in which the organisms were grown enabled growth of *A. acetabulum* from the juvenile phase through gametangial formation in 11 weeks at high population densities without medium replenishment (Fig. 8 in Hunt and Mandoli, 1996). This represented a 98% reduction in the seawater volume required to mature each individual, a 30–40% reduction of the duration of the life cycle (Fig. 5), an estimated 80% reduction in labor, and a 50–95% reduction in the space required for culturing *A. acetabulum* compared with traditional procedures (Hunt and Mandoli, 1996). Further improvements in the medium, i.e., removal of heavy metals from the medium, only marginally improved the rate of cap initiation in our artificial seawater, Ace-25 (Nguyen and Mandoli, 1998). The vitamin requirements for cap initiation have not been quantitatively determined. Not surprisingly, in this seawater, the temperature and light environs also affect cap initiation. The optimum photon flux density for cap initiation is from 150 to 250 μM m^{-2} s^{-1} for cool white light and the temperature optimum for cap initiation is from 19 to 21°C (Kratz and Mandoli, 1998b). At a comparable value (225 foot candles of fluorescent light), optimal growth was obtained for 8- to 16-h days for *A. crenulata* in a different seawater recipe (Terborgh and Thimann, 1964). These improvements in the growth conditions for the species have largely obviated the past problems in obtaining reliable populations of individuals which initiate caps.

The second problem in obtaining cap initiation was that, like other algae (M. Taylor, personal communication), this species is particular about the physical environs in which it grows. For unclear reasons, reproductive onset was <7% in populations of heterogeneous, wild-type strains grown in cer-

tain containers such as polystrene culture flasks (Table III). Although algae were sensitive to certain types of plastics (Zeller and Mandoli, 1993), this container preference seems to have to do with the container shape and the surface to volume ratio more than it does with the materials from which the container was made (B. E. Hunt and D. F. Mandoli, unpublished observations). *A. acetabulum* grew poorly in either new or used round petri dishes made of glass or polystrene: they remained vegetative and frequently died (Table III; B. E. Hunt and D. F. Mandoli, unpublished observations). Fortunately, algae grew quickly from one whorl to cap initiation in populations at 2.5 ml of Ace-25 seawater per individual, as long as the algae were grown in square, *not round,* containers made of polystrene with a surface to volume ratio >1.60 (B. E. Hunt and D. F. Mandoli, unpublished observations). Under these conditions, 95–100% of the individuals initiated caps within about 50–60 days (Nguyen and Mandoli, 1998). While the solution to this somewhat bizarre problem is far from intellectually satisfying, it is practical. In sum, the physical environs for obtaining cap initiation are no longer a problem as long as one is aware of the issue and knows how to circumvent it.

D. Completion of Reproduction

The apex of the alga undergoes a terminal differentiation during the completion of reproduction. The basic tripartite structure of the cap is defined within slightly less than 3 days after the formation of the knob-shaped stalk apex (Kratz *et al.,* 1998). At the stalk apex a series of shape changes ensues after the knob-shaped apex, which results in the formation of a mature cap (Kratz *et al.,* 1998). The gametophores of the cap remain connected to the stalk until very late in the completion of reproduction because chloroplasts move from the stalk into the cap throughout cap expansion. It is not known whether the vacuole or portions of it move out of the stalk while chloroplasts are migrating into the cap. The final structures generated by the apex are four to six sets of hairs which arise from the innermost lobe of the cap and are initiated roughly 2 days after knob (Kratz *et al.,* 1998). However, when the gametangia form the cap hairs, the coronae and the stalk are all clear, i.e., devoid of chloroplasts and perhaps of cytoplasm, which suggests that later in reproduction the coronae are walled off from the gametophores. The mechanism of pattern formation at the stalk apex (Harrison, 1994) and how the shape changes within the apex are generated are unknown.

1. Uses of the Reproductive Phase

Sexual reproduction may be the only means that this alga has to propagate the species since no means of reliably inducing vegetative propagation

have been determined. Puiseau-Dao (1962) postulated that the vegetative organism retracts its cytoplasm during the winter and grows a new stalk and a cap only during the second year. However, to the best of my knowledge, the very reasonable idea that individuals live vegetatively for more than 1 year in the wild before they reproduce has actually never been demonstrated in populations grown in the ocean. One observation hints that mitosis may, on occasion, precede meiosis. In the rare instances when the stalk branches (Bonotto, 1988; Mandoli *et al.,* 1995) a cap is made on each stalk apex so that the branched organism has two or more caps. In such individuals, gametangia initiate at different times in each cap. Clearly, there is nuclear material left in the rhizoid that is used to fill second and subsequent caps, but the ploidy and number of these postulated nuclei are unknown. Clearly, the ability to propagate the organism vegetatively would be a valuable tool.

The events in the completion of reproduction lend themselves to at least three other areas of interest to modern biology: the role of the cytoskeleton in development, the control of mitotic proliferation during unusual types of cell cycles, and as a biochemical source for the enzymes in DNA synthesis and nuclear movement. Current progress on the cytoskeleton of *A. acetabulum* is encapsulated in a recent review (Menzel, 1994). Except for some chromosome staining (De and Berger, 1990) and chromosome spreads (Spring *et al.,* 1975), aspects of the cell cycle have barely been examined in any *Acetabularia* species. For example, it is not known if individuals in the vegetative phase are in G_0 or G_1. There are some developmental studies of the timing of the onset of DNA synthesis but the proliferation of the DNA has not been used as a source for biochemical studies per se.

The events which occur during the completion of reproduction are conducive to teaching many principles of cell biology. For students of all ages, this is a large example of a "cell" and the large and complex cytoarchitecture makes many principles of biology easy to examine (Mandoli, 1993). For more advanced students experimentation on these organisms is also very appealing. For example, one can simply wound vegetative algae and watch them heal (Fester *et al.,* 1993; Mandoli, 1993), but during nuclear transport, reproductive algae do not heal well if at all. Repeating some of the classical amputation and grafting experiments is not beyond students in college courses. During the reproductive phase nuclei are visible and it is easy to watch their transport.

2. Challenges with the Completion of Reproduction

Two problems with culture of this portion of the life cycle are that heterogeneous wild-type strains abort reproduction (Runft and Mandoli, 1996) and that plants in a population undergo gametangial formation fairly asynchro-

nously. Abortion occurs at a low frequency, only 4–6% of the population, in two heterogeneous wild ecotypes (Runft and Mandoli, 1996) and so is not a major problem for culture. Individuals that have aborted will often succeed in completing reproduction with the second or third cap formed. While the asynchrony of gametangial formation is a nuisance (Geil and Mandoli, 1998b), it is only a major problem if this is the target of study. The nutrient conditions, specifically the presence of cationic transition metals in the seawater, impact completion and the synchrony of formation of viable gametangia. If some of the prestocks for the seawater are stripped of transition metals over a cation exchange column, the rate of gametangial formation increases and the viability of the population improves markedly (Nguyen and Mandoli, 1998). However, which cations are actually being removed or balanced is not known. Populations of inbred lineages are more synchronous in gametangial initiation and tend to produce higher percentages of individuals with gametangia than do heterogeneous wild-type lineages (B. E. Hunt and D. F. Mandoli unpublished observations). In practice, neither of these two problems with completion of reproduction impedes culture to an appreciable extent relative to the issues during other portions of the life cycle.

IV. Concluding Remarks

Will *A. acetabulum* experience a renaissance? In my opinion, a resurgence will rest largely on three factors: easy culture, access to genetic analyses including the ability to clone the relevant genes, and a "critical mass" of researchers. Although pedestrian in nature, streamlined culture methods are fundamental to any model system. They are, however, not trivial to develop and do not tend to result in flashy publications. Fortunately, the remaining problems with *A. acetabulum* hamper culture but none preclude it. The ability to do genetic analyses will have a major impact on the long-term viability of the system. Although *A. acetabulum* lacks a database in genetics, large-scale mapping of conserved regions of genomes between organisms may well speed development of genetic information because it is no longer necessary to collect information gene by gene. Such cross-genomic databases will probably not supplant the need for model systems for assessing gene function *in vivo* (Strauss and Falkow, 1997), at least for a long time. Finally, attracting a critical mass of people to the system will ensure a pace and level of excitement essential to make it competitive with more established model organisms. A community of researchers who enjoyed the benefits of open scientific communication was advocated by Jean Brachet (1970), a "dream" which was shared by Hans-George

Schweiger (Berger *et al.*, 1987) who died suddenly in 1986 (dedication in Berger and Kaever, 1992). Is this the lag phase before *Acetabularia* explodes in popularity once again? Only time will tell.

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Appendixes

It would be hard if not impossible to cite all the relevant references for this section since culture methods for the Dasycladales have been developed since the 1930s. Many papers have dealt with culture in general (Berger and Kaever, 1992; Hämmerling, 1931; Keck, 1964; Kloppstech, 1982; Lateur, 1963; Puiseux-Dao, 1962; Schmid and Giesecke, 1985; Schreiber *et al.*, 1964; Scheweiger *et al.*, 1977; Shephard, 1970; Shihira-Ishikawa *et al.*, 1985) for other opinions about how best to culture these species. Primary references for each of the specific methods that we use are given with each section of this appendix. For the sake of brevity, references cited in those primary references were not repeated here. Any oversights or omissions were not intentional and deserve apologia.

I. Cultureware and Media Preparation

Although prosaic, the basics of the cultureware and artificial seawater are essential to reliable, fast culture of A. acetabulum. *This seawater recipe has been tailor-made for this species and relies on highly purified water as a base. Deviations in the quality of chemicals or in the purity of the water used may be risky. We use a point-source water filtration system (Zyzatech Lab V) to produce our own HPLC-grade water for making artificial seawater* (Hunt and Mandoli, 1996) *and for all manipulations and biochemistry.*

A. Tool Maintenance

Seawater is highly corrosive, even to stainless steel, so poorly maintained tools do not last long. Corroded tools make sterile work less reliable.

1. Tool kit (per person):
   forceps, 2 pair fine
   dental tool, curved (GC-American Explorer No. ZMM0315, 3ES, AmflexII)
   dental tool, straight (Suter, 8.5 2 14, UWA No. 48-3)
   dental probe (Suter, Classic 5)
   scalpel for amputations
   scissors
2. Tool maintenance kit for metal tools:
   sandpaper, coarse emery (Norton, fine No. 084)

sandpaper, fine emery (Norton, 400-A, 423, T421)
—mineral oil

Rinse off your tools with fresh water to remove any medium after each use.

Dry tools and use fine sandpaper to remove the minor rust and debris that result from daily usage. If the tools are badly corroded, use coarse sandpaper to smooth the surface.

Remove metal dust with a Kimwipe.

Coat tools in mineral oil. Remove excess oil with Kimwipe or paper towel.

NOTES: Store your scissors in the open position; if they do rust they will rust in the open position and be easier to repair. The impact of corroded metal from tools on growth of the species has not been assessed but may be minimal given the length of time the tools are in contact with the medium. Although keeping tools free of corrosion is mainly to preserve the life span of the tools themselves, it is possible that using clean tools limits inadevertant contributions of metals to the medium as well.

B. Sterilization of Culture Dishes

Glass cultureware is more expensive and heavier than plasticware. In addition, glass can be a hazard when it breaks. Polycarbonate and polystrene plasticware can be used multiple times without adverse effects on growth or reproduction. In our experience A. acetabulum reproduces better in square rather than round containers (B. E. Hunt and D. F. Mandoli, unpublished observations) *and square glass petri dishes are not available.*

1. Autoclaving Polycarbonate Boxes (for 100–150 Plants per Box)

Autoclave clean, polycarbonate boxes (10.9 cm square with lids Sigma Catalog No. C8062) for 20 min at 121°C at 15–20 psi on dry cycle.

2. Gas Sterilizing Polystrene Petri Dishes (Zeller and Mandoli, 1993) (for 20 Plants per Dish)

Pack clean, dry polystyrene petri dishes (Nunc) in thin, unwaxed brown paper (15 × 9 × 28 cm).

Treat with ethylene oxide (gas) in a certified facility. Hospitals gas-sterilize surgical supplies since gas is nonpyrogenic. This neurotoxin requires 24 h of detoxification at the licensed facility.

Out gas in a ventilated hood for 2 months before use. This removes the last traces of gas from the plastic. This precaution with ethylene oxide use with polystrene is necessary to meet health and safety levels for this neurotoxin (R. Easterling, B. Hunt, and D. Mandoli, unpublished observations).

NOTE: Microwaving plasticware failed to consistently sterilize dishes (R. F. Kratz and D. F. Mandoli, unpublished observations).

C. Dish Washing for Algal Culture

1. For Dishes Used for Chemicals

Use 95% EtOH to remove pen labels from dishes. Used EtOH is okay. Be sure to do this step first as the ethanol leaves streaks on the dishes.

Wash dishes 1× in tap water and 3× in distilled water (purified by reverse osmosis).

2. For Dishes in Which Algae Have Been Grown

Use a wet sponge to carefully wipe the bottom and sides of plant culture dishes. Secretions, salt, and debris do not come off with rinsing alone.

Acid wash with 0.12 *N* HCl those dishes which have white spots or which contained microalgal, fungal, or bacterial contaminants. *A. acetabulum* makes a white substance which they may use to attach themselves to solid substrata. The acid wash dissolves this white substance.

Wash dishes 1× in tap water and 3× in distilled water (purified by reverse osmosis).

Let dishes air dry and pack them for ethylene oxide or gas sterilization.

NOTES: We no longer use any soap on our culture dishes. In the past we successfully used either RBS-35 (Pierce Chemical Co., Rockford, IL 61105) or 7X (ICN Biomedicals Inc., 3300 Hyland Ave., Costa Mesa, CA 92626). If you do not rinse both the insides and the outsides of the dishes with distilled water 3×, water spots will form when the dishes dry. Water spots are caused by salts in the tap water that become concentrated as the water evaporates.

D. Synthetic Seawater [Ace-25 (Hunt and Mandoli, 1996) Based on Modified Müller's (Schweiger *et al.*, 1977)]

Although the inorganic components have been balanced, the inorganic components of this seawater have not been tested. Add solutions at the concentrations and in the order given since deviations may cause insolubility while

stocks are being mixed (e.g., pH changes during the preparation). Purified water was an essential step in developing this seawater so we have continued to use 18 MΩ purified water (Type I, HPLC grade) exclusively to make all our solutions and our seawater.

Adjust pH to 7.8 with 10 m*M* NaOH or ≤12.1 *M* HCl. Higher concentrations of NaOH will precipitate seawater but any concentration of HCl can be used.

Add 100 μl/liter of 10,000× sterile vitamin stock either just before use OR before sterile filtration.

Filter sterilize (see equipment and protocol) and store at 4°C.

	To make a final volume of x liters, add these stock volumes (milliliters)				
Chemical	*50 L*	*25 L*	*20 L*	*1 L*	*Comments*
H_2O (18 MΩ, HPLC grade)	45,000	20,000	18,000	800	
$NaCl$-Na_2SO_4	—	—	—	—	Dry, see below
CO_3-NO_3-K-EDDHA:Fe	500	250	200	10	
$MgCl_2$-$CaCl_2$	1056	528	422	21	See below
EDTA-Mn-Zn-Mo-Cu	50	25	20	1	pH ≥ 7.0
B-PO_4-Li-I	50	25	20	1	
Si	50	25	20	1	pH ~ 2.0
Vitamins	5	2.5	2.0	0.1	

E. Stocks for Synthetic Seawater (Ace-25)

The following stock and prestock solutions have been developed to minimize labor inherent in making the artificial seawater, Ace-25. The nine final stocks have been carefully designed to minimize adjustments in pH and to maximize solubility of the individual stocks as well as the final seawater.

Use 18 MΩ water (HPLC grade, Type I) for all the stock solutions.

Autoclave each inorganic stocks for 30 min at 120°C and 15–20 psi. Filter sterilize vitamins.

Store all of the autoclaved stocks at room temperature. Store vitamin stock at −20°C.

1. Dry Chemical Mixture

It is most labor efficient to weigh out the dry amounts of these two salts rather than dissolve a stock solution because they are so concentrated in the final seawater. If you prefer, weigh out lots of these two salts and store them dry until use. The more expensive grade sodium dissolves better and gives the least problems with precipitation so it is cost effective overall.

	Concentration, final (mM)	Dry Chemical Mixture (grams) for 50 L	25 L	20 L	1 L
Na_2SO_4 (anhydrous)	5	35.51	17.75	14.21	0.71
NaCl	525	1534.12	767.06	613.60	30.68

2. CO_3-NO_3-K-EDDHA:Fe Stock (100×)

This stock provides carbon, nitrogen, potassium, and iron for growth. It relies on one prestock which is needed to prechelate the iron to EDDHA.

Chemical	Concentration final (x)	Weight for 200 ml	liter	Instructions
$NaHCO_3$	2.4 m*M*	—	20.16 g	
$NaNO_3$	3 m*M*	—	25.50 g	
KCl	10 m*M*	—	7.46 g	
EDDHA:Fe	1.0 μM:1.6 μM		10 ml	of 10,000× prestock

FE-EDDHA prestock (10,000×) *EDDHA, the second chelator in this seawater, is specific for iron. It was needed to solubilize enough iron for growth and reproductive onset at the alkali pH of the seawater.*

Combine the ingredients and bring to a pH ≥ 8.0 to solubilize the EDDHA (ethylenediamine di(o-hydroxyphenylacetic acid).

Adjust the pH with concentrated NaOH liquid (for 200 ml) or pellets (for 1 liter).

Sparge (bubble with air vigorously) overnight to drive the chelation. The finished prestock will be a deep, rich red and the pH will drop to ≥7.

Chemical	Concentration final (X)	Concentration Prestock	Weight for 200 ml	Weight for 1 liter
NaOH	—	—	As needed	5.00 g
EDDHA	1.0 μM	10 m*M*	0.72 g	3.60 g
Fe-citrate	1.6 μM	16 m*M*	0.78 g	3.92 g

3. Magnesium and Calcium Stock

We find it easiest to make magnesium and calcium chloride stocks in the original shipping bottles. The magnesium and calcium chloride solutions are

made separately and then mixed together to make the final stock solution. This is necessary because the salts are so hygroscopic that one cannot accurately adjust the concentration, i.e., weigh them out, otherwise.

Add enough water to the shipping bottles of $MgCl_2$ and $CaCl_2$ to solubilize the chemical.

Adjust the concentrations of the these solutions by refracting the $MgCl_2$ to $n_D = 1.38364$ and the $CaCl_2$ to $n_D = 1.36625$.

Mix 1.8 liters of refracted $MgCl_2$ and 0.7 liter of refracted $CaCl_2$ and use as the stock solution.

		Weight for		
Chemical	*Concentration, final (x)*	*200 ml*	*Liter*	*Instructions*
$MgCl_2 \times 6\ H_2O$	35 m*M*	—	467.4 g	$MgCl_2\ n_D = 1.38364$
$CaCl_2 \times 2\ H_2O$	8 m*M*	—	198.4 g	$CaCl_2\ n_D = 1.36625$

a. Pre-Stocks for Chelated Trace Metal and B-PO_4-Li-I Stocks *The following five very concentrated pre-stocks enable each of these chemicals to be weighed accurately. Use these pre-stocks to make the stock solutions (#4 and 5). Finally, the stock solutions, not the pre-stocks, are used to make Ace-25 seawater.*

Lithium chloride is hygroscopic. Use a weigh boat rather than paper to measure it. Then, if it is wet after you weigh out the correct amount, you can just rinse it into the rest of the prestock solution.

	Concentration		*Weight for*	
Chemical	*Final, (x)*	*Prestock*	*1 ml*	*10 ml*
$MnSO_4 \times 1\ H_2O$	0.1 μM	0.1 *M*	15.1 mg	151 mg
$Na_2MoO_4 \times 2\ H_2O$	0.05 μM	0.05 *M*	10.3 mg	103 mg
$CuSO_4$	1.0 n*M*	1 m*M*	0.25 mg	2.5 mg
LiCl (hygroscopic)	0.2 n*M*	0.02 *M*	0.85 mg	8.5 mg
NaI	0.044 μM	0.044 *M*	6.6 mg	66 mg

4. Chelated Trace Metal Stock (1000×)

The metals other than iron are best chelated by the relatively nonspecific chelator EDTA.

Dissolve the EDTA in water (18 MΩ).

Bring the pH to ≥7.

Add the rest of the metals in the order given to make the 1000× stock.

Chemical	Concentration, final (x)	Weight for 200 ml	Weight for Liter	Instructions
$Na_2EDTA \times 2\ H_2O$	2.75 μM	205 mg	1.07 g	
$MnSO_4 \times 1\ H_2O$	0.1 μM	200 μl	1 ml	Use prestock
$ZnCl_2$	1.0 μM	27.3 mg	136 mg	
$Na_2MoO_4 \times 2\ H_2O$	0.05 μM	200 μl	1 ml	Use prestock
$CuSO_4$	0.001 μM	200 μl	1 ml	Use prestock

5. B-PO_4-Li-I and Si Stocks (1000× Each)

Because the silica stock is very acidic, we found that it could be not be combined with any other stocks without causing solubility problems.

Chemical	Concentration, final (x)	Weight for 200 ml	Weight for Liter	Instructions
H_3BO_3	100 μM	1.24 g	6.18 g	
Na_2HPO_4 (anhyd.)	50 μM	1.42 g	7.1 g	
LiCl	0.2 nM	2 μl	10 μl	Use prestock
NaI	0.044 μM	200 μl	1 ml	Use prestock
$Na_2SiO_3 \times 5\ H_2O$	70 μM	2.97 g	14.85 g	Acidic; keep separate

6. Vitamin Stock (10,000×)

The vitamins are the only part of this seawater for which concentration curves have been done.

Dissolve all vitamins together in water.

Filter sterilize.

Aliquot into sterile tubes as desired (some combination of 10 ml and 1 ml are convenient).

Store at −20°C.

Chemical	Concentration, final (x)	Weight for 200 ml	Weight for Liter	Instructions
Thiamin-HCl	300 μg/liter	600 mg	—	Filter sterilize
p-aminobenzoate	20.0 μg/liter	40 mg	—	Aliquot and store at −20°C
Ca-pantothenate	10.0 μg/liter	20 mg	—	
Biotin	5.0 μg/liter	10 mg	—	
Cyanocobalamine, B-12	4.0 μg/liter	8 mg	—	

F. Components in Artificial Seawater Media[a]

Ion	*Ace 25 (Hunt and Mandoli, 1996)*	*Müller's (Schweiger et al., 1977)*	*MCM (Woelkerling et al., 1983)*	*ESAW (Harrison et al., 1980)*	*Shephard's (Shephard, 1970)*	*ASP6 (Sigma Chemical)*
Macronutrients, m*M*						
Cl^-	578.00	521.00	505.00	427.00	428.00	424.00
Na^+	556.00	479.00	475.00	390.00	412.00	415.00
Mg^{2+}	35.00	49.60	40.00	47.20	48.70	32.50
Ca^{2+}	8.00	13.50	5.00	9.10	6.80	3.75
K^+	10.00	9.80	10.00	8.00	10.00	9.40
HCO^{3-}	2.40	2.40	2.05	2.10	1.19	—
NO_3^-	3.00	1.18	1.00	0.55	0.47	3.50
SO_4^-	5.00	26.60	1.00	25.00	48.70	32.50
Micronutrients, μM						
PO_4^{3-}	50.00	140.00	30.00	21.80	5.70	0.46
Fe^{3+}	1.60	1.20	1.00	6.56	1.80	35.80
Mn^{2+}	0.10	3.80	1.00	2.42	1.00	1.80
Zn^{2+}	1.00	14.00	1.00	0.25	7.00	7.70
MoO_4^{2-}	0.05	0.83	1.00	—	4.10	5.20
BO_3^{3-}	100.00	32.00	1.00	0.43	—	185.00
Cu^{2+}	0.001	0.008	0.30	—	0.008	0.32
Co^{2+}	—	0.04	0.20	0.06	0.008	0.17
Micronutrients, μM						
Li^+	0.002	0.14	—	—	—	—
Rb^+	—	0.13	—	—	—	—
Si^{2+}	—	14.00	—	82.00	—	—
Al^{3+}	—	0.21	—	—	—	—
I^-	0.044	0.12	—	—	—	—
Br^-	—	185.00	—	725.00	—	—
SiO_3^{2-}	70.00	70.00	—	105.60	—	246.00
F^-	—	—	—	65.70		—
NH_4^+	—	—	—	5.97	—	—
Chelators and buffers, μM						
EDTA	2.88	53.70	6.60	14.86	35.70	—
EDDHA	0.60	—	—	—	—	—
Tris	—	—	660.00	—	8.30	8.30
Vitamins, n*M*						
Thiamine-HCl	890.00	890.00	1480.00	297.00	890.00	6000.00
Biotin	20.00	20.00	4.00	1.47	—	20.00
Cyanocobalamin	3.00	3.00	0.74	4.09	3.00	0.37
Ca-pantothenate	42.00	42.00	—	—	42.00	4000.00
PABA	140.00	140.00	—	—	150.00	0.73

[a] These values were calculated based on the amounts of the reagents used to make up these media and do not necessarily reflect the chemical species in each of the final seawaters.

G. Sterile Filtration of Large Volumes of Seawater

1. Before You Start

Before you assemble the filtration rig be sure that the O-rings have a light coating of vacuum grease and that the filter is dry all the way through the center. The filter will not be sterilized if it is wet.

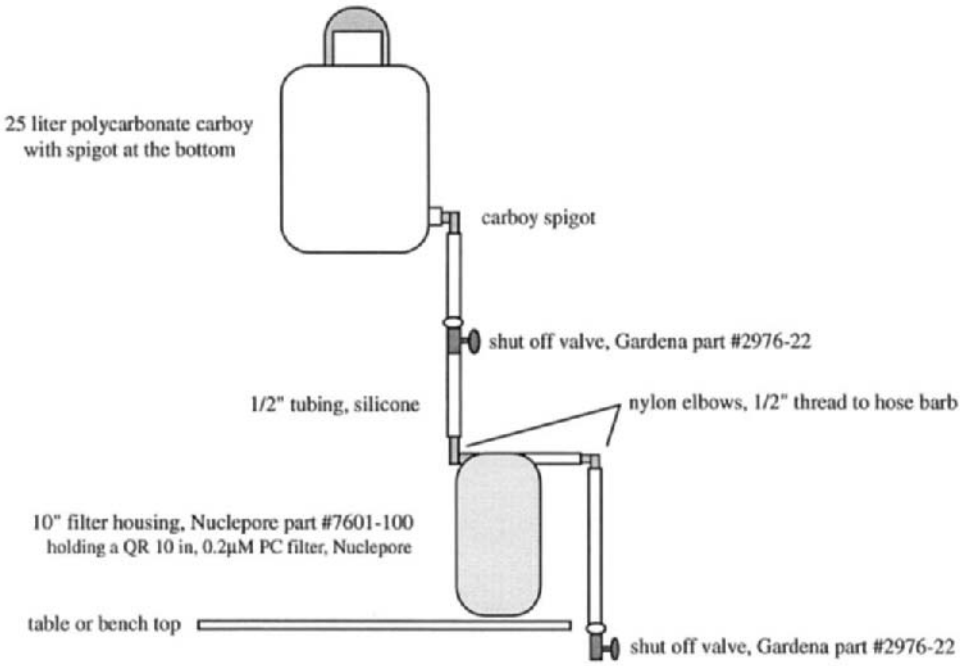
Assemble and gas the filtration rig. Be sure the valves are open.

Sterilize all the containers to hold the media. A few extra is a good idea. We use 1-liter orange top flasks, Pyrex No. 1395, to store our seawater.

Make 50–200 liters of seawater.

Make LBS plates. Autoclave pipet tips to test the media (we use 5 μl per patch per container).

2. Equipment Diagram



For Nuclepore parts contact: Corning Costar One Alewife Center, Cambridge, MA 02140 1-800-492-1110

3. Filtration Setup

Number the containers. Arrange them in sets of 5–10 numerically within easy reach.

Add vitamins to the seawater.

Stir carboy of media before filtration to ensure that the vitamins are well mixed.

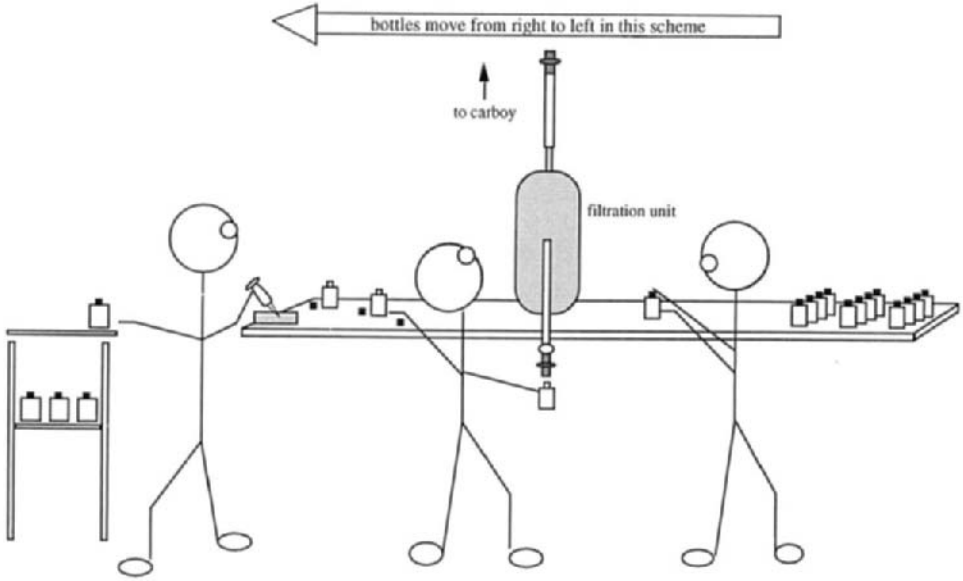
Put the carboy on a shelf so that the end of the nozzle is 2.5 feet above the top of the pair of filtration cannister. Open the top of the carboy.

Close both shut-off valves.

Attach the hose that leads to the filtration housing to the carboy nozzle. If you wish, you can secure the hose with a 5″ electrical tie.

Open the carboy nozzle.

4. Filtration of Large Volumes of Seawater



5. Filter Rig Cleanup

Open the filter rig. Rinse the hoses and filter with distilled water until the water does not taste salty.

Fill the housing which contains the filter with distilled water and add a small amount of bleach (just until you can smell it) and let the housing sit for 0.5 h.

Rinse the housing thoroughly until you can no longer smell the bleach and then let it air dry. It will dry much faster if you remove the filter from the cannister.

6. Check Your Filtered Product

Incubate 5- to 10-μl aliquots of the filtered medium on LBS at room temperature for 5 days.

Discard media that is contaminated. Retest any bottles of media that give ambiguous results.

H. Sterilization of Small Volumes of Seawater

1. The Day before You Want to Filter Media

Autoclave filter housing (Nalge Co. part no. 320, polysulfone reusable bottle top filters, silicone O-rings, polypropylene adaptor for the vacuum port) and 1-liter bottles to hold medium (Corning no. 1395-1L, polypropylene plug to seal cap, polypropylene drip-free pour rings with 33- or 45-mm neck diameters as needed to fit your filter rig).

Wrap filters (Nalge Co. part no. 210-4020, 0.22 μm, 47-mm diameter, CA filter type) in aluminum foil. Prepare more than you need in case you tear one.

Bake the foil wrapped filters overnight at 80°C.

Make the medium you wish to filter sterilize.

Pour LBS plates to test your medium for sterility.

2. Small Filtration Unit Setup

Screw the filtration rig onto a Pyrex bottle. Set the Pyrex bottle top down onto a sterile surface.

Unscrew the upper and lower parts of the filter housing.

Flame blunt-tipped forceps and gently put the filter onto the lower portion of the filtration rig.

Screw upper part of the housing back onto the lower part, sandwiching the filter in between the two halves of the housing. Avoid touching the inside of the housing. If the filter rips, discard it.

Wet top of filter with 18 MΩ water.

3. To Filter Small Volumes of Seawater

Turn on vacuum or suction source.

Add medium to be filtered into the upper portion of the filtration rig. Replace the lid of the filtration housing.

Attach the vacuum hose to the filtration housing that you just filled with medium.

Continue filtering medium until the filter clogs (~5–6 liters), replacing bottles as needed.

Unplug the vacuum source BEFORE you turn off the vacuum to prevent backflow.

Clean housing and discard the used filter.

4. Check Your Filtered Product

Incubate 5- to 10-μl aliquots of the filtered medium on LBS at room temperature for 5 days.

Discard media that is clearly contaminated. Retest any bottles of media that give ambiguous results.

I. Agar Media Recipes

1. Luria–Bertani Broth (Kennedy, 1971) in Seawater ("LBS")

Use for storage of caps and checking contamination of Acetabularia caps.

Mix well:

10 g	Bacto-tryptone
5 g	yeast extract
7 g	Bitek or Bacto-agar (Difco)
800 ml	artificial seawater

Optional: Heat in microwave to dissolve agar.

Bring to 1 liter with artificial seawater.

Autoclave at 15 psi in a 2-liter flask covered with foil for 30 min on a liquid sterilize cycle with 0 min of dry time.

Immediately after autoclaving, swirl the flask gently to redistribute agar.

Cool until almost comfortable to hold, 50°C, and then pour into petri dishes.

Flame the surface of the agar immediately with a Bunsen burner to remove bubbles.

Cure for 2 days, agar-side-up at room temperature until plate surface dries out a little.

Either wrap each plate with parafilm *OR* put unwrapped plates in a tightly sealed box.

Store at room temperature if plates will be used within 14 days. Store at 4°C if they will be used after >14 days.

2. Seawater–Agar Plates (Hunt and Mandoli, 1992)

For gamete release and zygote growth of Acetabularia on a hard surface

Mix:

7 g	SeaPlaque agar (FMC Corporation).
1 liter	artificial seawater (with or without vitamins)

Autoclave at 15 psi in a 2-liter flask covered with foil for 30 min on a liquid sterilize cycle with 0 min of dry time.
Immediately after autoclaving, swirl the flask gently to redistribute agar.
Cool until almost comfortable to hold, 50°C, and then pour into petri dishes for use within 1–2 days
OR store in the flask at room temperature and aliquot aseptically at a later date.
Optional: Spread a 1/10 dilution of sterile vitamins onto the plates just before use.

3. Seawater–Agar Liquid (Mandoli and Larsen, 1993)

For gamete release and zygote growth of Acetabularia in a liquid matrix.

Follow the instructions for seawater–agar plates but reduce the amount of SeaPlaque agar to 1 g per 1 liter of seawater.

Store 0.1% seawater agar at room temperature. We autoclave 50-ml aliquots in 100-ml Pyrex bottles and tighten down the lids for storage after the agar has cooled.

Add vitamins to the matrix just before use.

II. Culture and Genetics

The most important aspect of culturing A. acetabulum is to maintain high-quality physiological conditions. First, high-quality artificial seawater made in a pure water base is essential for the best physiology. Second, if the correct population density for each phase of development is not obtained, the plants will not grow, not differentiate, or will die outright.

A. Inoculations of Vegetative *A. acetabulum*

Again, many problems in culture can be attributed to a failure to maintain the population density appropriate for the developmental phase of the organism.

1. Conditions for Phase-Specific Culture

Inoculation of	*Initial morphology*	*Population density*	*Goal of culture step*
Zygote	0.1-mm sphere	$\geq 10^4$ algae/ml	Zygote differentiation
Juvenile	1 whorl per alga, <1 cm in length	10^2 algae/ml	Juvenile growth

Inoculation of	*Initial morphology*	*Population density*	*Goal of culture step*
Adult	>6 whorls per alga, >1 cm in length	25 algae/50 ml	Adult growth and gametangial production
Single, adults	>6 whorls per alga, >1 cm in length	1 algae/20 ml	Cell biology and other experiments

Notes: The values in this table are for use with Ace-25 seawater medium.

2. Zygote Cultures (Cooper and Mandoli, 1998)

Make a 10% dilution of the zygote stock, e.g., inoculate 5 ml of zygote stock into 45 ml of seawater in a sterile 50-ml Falcon tube.

Incubate at 21°C with 120–170 μM m^{-2} s^{-1} cool white light with the tube lying on its side, for 6 days. Rock the tube from end-to-end to "discourage" them from attaching to the walls of the tube.

3. Juvenile Inoculations (Zeller and Mandoli, 1993)

When the algae have begun to produce whorls, inoculate into seawater (or test media).

Pour about half of the nursery culture into a small petri dish.

Using a sterile Pasteur pipette or an inoculating loop count the number of individuals as you pick them up.

Expel the algae into the seawater. Rinse out any individuals that are stuck on the inner walls of the pipette by aspirating and expelling some of seawater from the dish into which you are inoculating.

Seal the dish with one layer of parafilm.

OR, if you do not need a culture which is synchronous in growth and development, just dump the entire culture into a larger petri dish, allow them to grow, and thin them later.

4. Thinning for Adults and Gametangia (Hunt and Mandoli, 1996; Nguyen and Mandoli, 1998)

When the algae are approximately 1 cm (or large enough to see by eye) count the individuals and thin as necessary. Grow large populations en masse *in polycarbonate boxes and small populations in square petri dishes.*

Use an alcohol-flamed inoculating loop or dental tool to pick up individuals.

Put extra algae into a square petri dish containing sterile seawater.

B. Cap Harvest and Gametangial Maturation

Remove individual plants with clear stalks that have gametangia-bearing caps. Option for very dirty cultures: Rub each cap on clean Kimwipes until no more microalgae and epiphytes come off. These will feel slimy to the touch whereas a cleaned cap will feel smooth but not slimy. Use a fresh, dry Kimwipe for each cleaning.

Cut off stalks at the cap junction. Save the stalks for clearing and analysis if desired.

Incubate in synthetic seawater + vitamins (5 μl of 10,000× sterile stock/50 ml of seawater) at 4 ml per cap for 3–5 days at 21°C on a 14:10 L:D cycle.

Decontaminate *Aa* 0006 (a.k.a. Ladenburg no. 5) after 3 days and *Aa* 0005 (a.k.a. Ladenburg no. 17) after 5 days.

C. Decontamination of Matured, Gametangia-Bearing Caps

1. General Method for Decontamination (Hunt and Mandoli, 1992; Mandoli *et al.*, 1995; Shephard, 1970)

Incubate 5–30 caps/tube (the number of caps/tube depends on how large the caps are and what you need them for; if they are precious, use fewer caps/tube since the pipet tips tend to crush and break the cap walls when the caps are densely packed) in a 1.5-ml Eppendorf tube for 1 h at room temperature with 20 Anson units/ml Proteinase K or pronase E dissolved in digestion buffer. Use enough so that the entire surface of each cap is covered by the enzyme cocktail, usually about 0.1–0.3 ml/cap. Agitate at 50 rpm during the incubation with the tubes on their sides.

Wash 1× in 1 ml sterile, 525 m*M* NaCl.

Incubate the caps in the dark in either (a) 0.3% silver protein in sterile, 525 m*M* NaCl (3 mg/ml) for 5 min or (b) 0.01% silver protein (0.1 mg/ml) for 30 min.

Wash in 1 ml sterile, 525 m*M* NaCl either (a) 5× if you used 0.3% or (b) 3× if you used 0.01% silver protein.

Incubate in the dark in the antibiotic solution (below) for 3–5 days at room temperature (21°C).

Wash 3× in 1 ml sterile 525 m*M* NaCl.

Array caps in a patch pattern on 0.7% LBS plates. Parafilm the edge of the plate.

Incubate the caps on the plate with the agar-side-up for 3–5 days at room temperature.

Check for contaminants. Microalgae can be removed by gentle scraping or wiping on a clean agar surface. Transfer the clean caps to a fresh LB

plate. If microalgae still do not come off, discard the cap—you are not likely to be able to save it.

NOTES: Using this procedure, we routinely obtain 56–100% cap viability with <20 microalgal cells/cap with 0–5% fungal and bacterial contamination.

2. Stocks for Decontamination (Hunt and Mandoli, 1992; Mandoli *et al.*, 1995; Shephard, 1970)

Note that the antibiotics have not been individually titrated for the decontamination of A. acetabulum *caps but the SDS and buffer concentration have been optimized.*

a. Antibiotic Solution (Gibor and Izawa, 1963; Hunt and Mandoli, 1992; Shephard, 1970) (for 10 ml Total) *You can easily scale this up to make 50–100 ml at a time.*

[2 mg chloramphenicol—optional]
2 mg neomycin
20,000 units nystatin (e.g., 200 μl of 50× nystatin/10 ml antibiotic solution)
10 mg penicillin G, carbenicillin, or ampicillin
20 mg streptomycin
10 mLs sterile Ace-25 seawater

Store at −20°C.

b. Nystatin Prestock (50×) Calculate the final volume based on the concentration of the drug lot you purchased. Wet the powder in a small amount of dimethylsulfoxide (Sigma Bioscience, Plant Culture Catalogue 1996, Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178; Sigma provides a clear sample calculation.)

Suspend all the powder in the vial it came in with sterile water (18 MΩ, Type I, HPLC).

Aliquot 1 ml per Eppendorf tube using sterile technique.

Store at −20°C.

c. Digestion Buffer (Hunt and Mandoli, 1992) (for 100 ml Total)

10 ml 0.5 *M* BTP, pH 9.5 (use BTP Prestock below)
1 ml 10% SDS (9 ml H_2O/1 g SDS for 10% prestock)
89 ml sterile water (18 MΩ, Type I)

Store at room temperature.

d. BTP Prestock (for 100 ml Total of 0.5 M BTP)

14.12 g bis Tris propane
100 ml sterile water (18 MΩ, Type I)

Adjust pH to 9.5 with 12.1 *N* HCl.
Store at −20°C.

D. Gametangial Isolation (Keck, 1964)

1. Mechanical Method (Cooper and Mandoli, 1998; Shephard, 1970)

This method is useful for large numbers of caps which contain gametangia that are common, i.e., are just a laboratory strain (e.g., Aa*0005 or* Aa*0006).*

Decontaminate caps in a 50-ml Falcon tube through the Ag-protein step.

Grind the caps in the same tube in sterile 525 m*M* NaCl with hand-held homogenizer (Tissue Tearor, Model 985-370, Biospec Products Inc. from Whatman, P.O. Box 1359 Hillsboro, OR 9713-9981) until the caps are all broken up and the gametangia are free.

Strain through one layer of cheese cloth into a 15-ml Falcon tube.

Pellet the gametangia in a table-top centrifuge with a swinging bucket rotor at speed 1 or 2 for about 1–2 min or until they are pelleted.

Decant, add more 525 m*M* NaCl, and spin again. Repeat until the supernatant is clean of debris.

After the last spin, decant the supernatant and suspend the gametangia in the antibiotic cocktail.

Incubate for 3–5 days in the dark at room temperature with gentle shaking or rotating.

Spin down, decant supernatant, and rinse in sterile, 525 m*M* NaCl 3–5 times.

Store isolated gametangia in darkness at 15°C in seawater until needed (Hunt and Mandoli, 1998).

2. Manual Method

This method is useful for small numbers of caps which contain gametangia that are precious, that is, have been screened or selected for a particular phenotype or genotype and you do not have many of them. Perform all steps under a dissecting microscope using sterile technique either in a laminar flow hood or on a ethanol-cleaned benchtop, as you feel comfortable.

Make 100- to 200-μl puddles of sterile, purified water (18 MΩ, HPLC, Type I) in a sterile petri dish.

Add one axenic cap to each puddle using ethanol-flamed forceps.

Rip the gametangial rays at the center of a cap with an ethanol-flamed dental tool or with ethanol-flamed scissors.

Holding the outer edge of the cap with a dental probe, tease the gametangia into the center of the cap with a sharp, curved dental explorer.

Transfer the gametangia to 1 ml of sterile, purified water or sterile 525 m*M* NaCl in a sterile 1.5-ml Eppendorf tube.

Mix well to break up the clear mucilage in which the gametangia are embedded while inside the intact cap. Start another cap while gametangia from the last cap you worked with settle to the bottom of the tube OR pellet gametangia at 3000 rpm for 2 min.

Discard the 1 ml of liquid above the gametangia after all the gametangia have settled to the bottom of the tube.

Resuspend the gametangia in sterile, purified H_2O, 525 m*M* NaCl, seawater, *OR* in an antibiotic solution (Mandoli *et al.*, 1995).

Plate 5 μl of the resuspended gametangia onto an LBS agar plate to check for sterility either immediately if you resuspended in water, *OR* after an antibiotic treatment (3-day incubation, followed by 3× washes in 1 ml sterile, purified water or in sterile 525 m*M* NaCl).

Store isolated gametangia in darkness at 15°C in seawater until needed (Hunt and Mandoli, 1998).

NOTES: Some people in our lab prefer to work with just one cap a time on the petri dish and then move the gametangia from each cap to an Eppendorf tube. They wash all the tubes of gametangia as a separate step from gametangial isolation. Failing to wash off the mucilage will result in the gametangia being held in a stringy, proteinacious blob.

E. Mating Methods

1. To Assay Percentage or Kinetics of Gamete Release (Hunt and Mandoli, 1992; Mandoli and Larsen, 1993)

Suspend mature, axenic gametangia in either 0.1% Sea Plaque agar or sterile 525 m*M* NaCl. Use Sea Plaque if you wish to control the numbers of gametangia precisely.

Pick up gametangia either with a glass 5- or 10-ml micropipette attached to a sterile, cotton-plugged mouth aspirator or with a P-20 Gilsen pipetman. The micropipette will give you finer control.

Array groups of 10–20 gametangia in a patch pattern on 0.7% Sea Plaque seawater petri plates (60- or 100-mm-diameter round plates are fine).

Count gametangia immediately after plating, scoring total number of gametangia plated, the number of gametangia in that population which are green, dead, and empty shells.

Wrap the edges of the plates with parafilm. Incubate at 21°C, 120–250 $\mu mol^{-1}m^{-2}s^{-1}$ on a 14:10-h L:D cycle.

Count the number of gametangia which have released their gametes as needed.

Definitions of gametangial appearances:

Green: Dark or light green, rounded looking (as opposed to flat looking) with membrane in contact with the gametangial wall.

Dead: Plasmolysed (membrane retracted from the gametangial wall), brown or achlorotic balls inside gametangial wall.

Shells: Empty gametangia (clear walls) with operculum gone or open and still attached.

Gamete release: Operculum open and gametes swimming out. Gamete release takes 3–15 days.

2. To Mate Single Gametangial Pairs (Geil and Mandoli, 1998a; Mandoli and Larsen, 1993)

Put 200 μl of 0.1% SeaPlaque seawater–agar in each well of a 96-well microtiter plate or into a 0.5-ml Eppendorf tube. Flat-bottomed wells make it much easier to see the gametangia.

Put one gametangium from each individual you wish to mate into each well or tube. If you are doing a self-cross, put two gametangia from the same cap into each well.

Expect: gamete release in 1–15 days, depending on gametangial age and storage history; 1561 $\pm$ 207 zygotes per gametangial pair; and 95% of the resulting zygotes will be single, i.e., unattached to any of other zygotes.

3. To Mate for Large Populations of Algae Attached to a Solid Support (Mandoli and Larsen, 1993; Zeller and Mandoli, 1993)

Cut strands of orange-cotton embroidery floss (Dollsuf-Mieg & Cie no. 722) for the zygotes to attach to. Note that the length of the strands should be slightly shorter than the width of the container that the algae eventually will be grown in.

Untwist the six threads which comprise a strand of embroidery floss. Each thread should now consist of two, entwined filaments.

Label the threads with lettered beads if you wish. Positions along the thread also can be marked with colored "seed" beads spaced at known intervals. This helps in locating an individual of particular interest later on.

Sterilize the threads either with ethylene oxide or by autoclaving. Be careful not to melt the beads.

Inoculate with 100–1000 axenic gametangia/cm^2 in a 0.7% SeaPlaque seawater plate (see below). Take care to distribute the gametangia evenly. If the gametangia do clump, you will get clumps of zygotes which grow irregularly and slowly.

Expect: gamete release in 1–15 days, depending on gametangial age and storage history; 1561 ± 207 zygotes per gametangial pair on average; but only 0.1% mating efficiency with ~30% attachment of zygotes to these orange threads.

4. To Mate for Large Populations of Unattached Algae (Cooper and Mandoli, 1998; Hunt and Mandoli, 1998; Mandoli and Larsen, 1993; Mandoli *et al.*, 1995)

Inoculate with 10–1000 axenic gametangia into 4 ml of 0.1% SeaPlaque agar (see below). Rock to suspend the gametangia evenly.

Expect: gamete release in 4–21 days, depending on gametangial age and storage history; 1561 ± 207 zygotes per gametangial pair on average; and 100% mating efficiency with ≥90% of the zygotes unattached to any others.

III. Whole Cell Biology Methods

These are the amputation and grafting methods to which this organism is so well suited. Enucleation methods are given in publications by others.

A. Pressure-Wounding and Enucleation of Algae (Fester *et al.*, 1993)

Place a dissecting microscope in a laminar flow hood or a benchtop. Swab down both the microscope and the hood with 70% ethanol. Use sterile technique throughout the procedure.

1. To Pressure-Wound and Enucleate a Juvenile Alga

Flatten the stalk in one spot slowly, over 5–10 s, with the back of a sterile scalpel and hold the blade in place for 5 seconds.

NOTE: These two operations can be performed simultaneously on juveniles because their walls are thin and will adhere to each other when pressed together. Juveniles do not tend to lose cytoplasm as adults do.

2. To Pressure-Wound an Adult

	Time required to heal, minutes	
	Healthy alga	*Unhealthy alga*
Reseal vacuole	1.5–10	11–50
Striated chloroplasts	20–30	30–60

Place individual and 1–2 ml of sterile seawater into a 10-cm petri dish.

Locate the rhizoid of the individual under the dissecting microscope.

Flatten individual just above the rhizoid with the back of a sterile scalpel or a dental tool for 30 s. If the alga is cut at this point, try changing the angle of the blade and/or using less pressure when flattening the next one.

If you are not going to enucleate the individual, let it recover for 20 min at room temperature and normal room light. Place ligated alga back into the petri dish and resume normal growth conditions or continue with enucleation.

3. To Enucleate an Adult (Berger and Kaever, 1992; Borghi *et al.*, 1983; Keck, 1964; Mandoli and Hunt, 1996; Runft and Mandoli, 1996)

Slowly and gently cut through the middle of the ligated area with the sharp side of a new scalpel.

Do not move the amputee for several hours.

Resume normal growth conditions. You can now move the enucleated segment or change the media if desired.

NOTES: Leakage of cytoplasm can be minimized by holding the cut end above the rest of the alga with the scalpel until the leakage stops (<1 min).

B. Grafting Method (Berger and Kaever, 1992; Bonotto, 1989; Keck, 1964; Kloppstech, 1982; Mandoli and Hunt, 1996)

1. Materials Needed for Grafting

Sterile artificial seawater, Ace-25
Dental tool
Sterile Pasteur pipettes

Two pairs of fine forceps
Sterile petri dishes, 100 × 15 mm
Scalpel (Bard-Parke no. 3)
Scalpel blades (no. 15)
95% EtOH for sterilizing tools

2. How to Graft Two Fragments Together

Pour enough seawater into a sterile petri dish to just cover the bottom of the dish. Old petri dishes work best since they cause less problems with surface tension on the seawater.

Place the cells to be grafted into the petri dish. Choose cells that are not the same size in diameter in the regions which you wish to join together.

Adjust the volume of Ace-25 using a sterile Pasteur pipette so that there is only a thin film of seawater on the bottom of the dish and the cells do not float.

Alcohol-flame the dental tool and wound each cell by gently pressing straight down, or with a rolling motion if the cells are extremely turgid.

Wait for the cytoplasm to pull away from the wound site (about 5–30 s).

Alcohol-flame the scalpel blade and cut each individual into two pieces in the clear region of the wound site. If the cell wall sticks together at the cut site try to open it up by pinching it together in the opposite direction with the forceps. If the cut edges do not separate use another plant.

Alcohol-flame the forceps and use them to gently insert one plant inside the other so that the cut ends overlap just enough to hold the pieces together. Try not to crush the pieces with the forceps as you are manipulating them into position. If the wall of the inner plant is embedded in the cytoplasm of the outer one, the cytoplasm will contract and the graft seldom heals.

Allow the graft to heal for a several hours in dim light (normal room lighting). You can carefully slide the dish to the side about 5 min after grafting. To prevent evaporation of the media and plant death, carefully add more seawater to the dish about 1–2 h after grafting.

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The Role of the Dynactin Complex in Intracellular Motility

Elizabeth A. Holleran,* Sher Karki,* and Erika L. F. Holzbaur†
*Cell and Molecular Biology Graduate Group and †Department of Animal Biology, University of Pennsylvania, Philadelphia, Pennsylvania, 19104

Dynactin is a multisubunit complex that binds to the minus-end-directed microtubule motor cytoplasmic dynein and may provide a link between the motor and its cargo. Results from genetic studies in *Saccharomyces cerevisiae, Neurospora crassa, Aspergillus nidulans,* and *Drosophila* have suggested that cytoplasmic dynein and dynactin function in the same cellular pathways. p150^{Glued}, a vertebrate homologue of the *Drosophila* gene *Glued,* is the largest polypeptide in the dynactin complex with multiple protein interactions. Centractin, the most abundant dynactin subunit polypeptide, forms an actin-like filament at the base of the complex. Studies on dynamitin, the 50-kDa dynactin subunit, predict a role for dynactin in mitotic spindle assembly. Other subunits of dynactin have also been cloned and characterized; these studies have provided insight into the role of the complex in essential cellular processes.

KEY WORDS: Dynein, Dynactin, Actin-related proteins, Capping protein, Dynamitin, p150^{Glued}, Axonal transport, Nuclear migration.

I. Introduction

The directed motility of organelles along microtubules is a critical process involved in cell functions as diverse as vesicle transport and cell division. Our knowledge of the molecular motors which provide the force for these cellular processes is rapidly increasing (Sweeney and Holzbaur, 1996). Kinesin, the kinesin-related motors, and cytoplasmic dynein couple the energy of ATP hydrolysis to the production of vectoral force along microtubules. This force is in turn coupled to drive either the sliding of parallel microtubules or vesicular motility. Our knowledge of the mechanisms that

generate force and perform work is also growing. In this chapter, we review the role of dynactin in coupling cytoplasmic dynein to diverse cellular roles such as the assembly of the mitotic spindle, nuclear migration, and vesicle trafficking.

Dynactin, often referred to as an accessory factor of cytoplasmic dynein, is itself a complex molecule, consisting of subunits varying in size from 150 to 22 kDa. Initially, the 150- and 45-kDa subunits were identified as copurifying polypeptides in dynein preparations from liver and testis (Collins and Vallee, 1989). Subsequent biochemical analyses have revealed that these polypeptides are subunits of a large complex, along with 5 to 6 other polypeptides. Subunits of 150, 62, 50, 45, 37, 32, 27, and 22 kDa are found in a stoichiometry of 2:1:5:10:1:1:1:1 in the dynactin complex (Fig. 1; Holleran *et al.,* 1996; Schafer *et al.,* 1994a; Paschal *et al.,* 1993; Lees-Miller *et al.,* 1992; Gill *et al.,* 1991).

The structure of dynactin was determined by electron-microscopic analysis accompanied by immunolocalization studies using monoclonal antibodies specific for several of the dynactin subunits (Schafer *et al.,* 1994a). Dynactin was characterized by the following features: a 37-nm-long actin-like filament which forms the backbone of the complex and a 24-nm lateral projection which emerges from the short filament at an angle and terminates with two globular "heads." The actin-like filament is composed of the 45-kDa subunit of dynactin known as centractin or Arp 1, for actin-

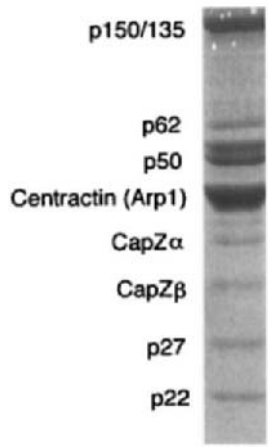

FIG. 1 Subunit composition of purified dynactin. Dynactin is an oligomer of several different polypeptides weighing in excess of 1 MDa. Shown here is a Coomassie-stained 12% SDS–PAGE of concentrated dynactin purified from an affinity column of a dynein intermediate chain (Karki and Holzbaur, 1995). Note that the p150/135 doublet does not resolve well on a 12% gel.

related protein 1 (Clark and Meyer, 1992; Lees-Miller *et al.,* 1992; Schafer *et al.,* 1994a). Centractin is the most abundant component of dynactin, with a stoichiometry of 8–13 monomers per complex (Paschal *et al.*, 1993; Schafer *et al.,* 1994a). The 24-nm lateral projection from this filamentous base is thought to be formed by the dimerization of two molecules of p150^{Glued}, which may be intertwined into a coiled coil with the amino-terminal domains projecting away from the base of the complex. The 37- and 32-kDa subunits of dynactin have been identified as isoforms of capping protein, or CapZ, and have been localized to one end of the centractin filament. Antibodies specific for the 62-kDa polypeptide bound to the other end of the centractin filament (Schafer *et al.,* 1994a). The binding sites for the 22-, 27-, and 50-kDa subunits have not been identified, but it has been suggested that the p50 (dynamitin) subunit is localized to the shoulder of the complex and may participate in anchoring the p150^{Glued} dimer to the centractin filament (Schafer *et al.,* 1994a).

Many of the subunits of the dynactin complex have now been characterized in molecular detail. No published sequence data are available yet for the 62-, 27-, and 22-kDa subunits of dynactin. However, molecular analysis of the p150, p50, p45, p37, and p32 subunits has provided significant information, both from analysis of the primary sequences and from overexpression studies in mammalian cells. These molecular analyses have also allowed direct comparisons with homologues in lower eukaryotes, where the phenotypes of null and disruption mutations have been characterized. The properties of each of the subunits are reviewed below, followed by comparisons of mutant phenotypes in yeast, filamentous fungi, *Caenorhabditis elegans,* and *Drosophila.*

II. Dynactin Structure and Composition

A. p150^{Glued}/p135

1. Molecular Characterization of p150^{Glued}

The p150^{Glued} subunit of dynactin is the largest polypeptide in the complex and was the first to be identified, cloned, and characterized. The 150-kDa polypeptide was initially identified as a copurifying protein in preparations of cytoplasmic dynein (Collins and Vallee, 1989). Using a polyclonal antibody to this polypeptide, Holzbaur *et al.* (1991) cloned cDNAs encoding p150 from a rat brain cDNA expression library. Database searches using the predicted amino acid sequence of this polypeptide showed significant relatedness (30% identity overall) with the product

of the *Drosophila* gene *Glued,* which had been previously cloned and sequenced by Swaroop *et al.* (1987). The name p150^{Glued} was hence adopted to describe the vertebrate polypeptide. The product of the *Drosophila* gene *Glued* was subsequently shown to be homologous in function as well as in sequence to the vertebrate polypeptide (Waterman-Storer and Holzbaur, 1996; McGrail *et al.,* 1995).

Predicted protein sequences for the p150^{Glued} polypeptide have now been obtained from human (Tokito *et al.,* 1996), mouse (Jang *et al.,* 1997), chick (Gill *et al.,* 1991), and *Neurospora crassa* (Tinsley *et al.,* 1996). The human gene encoding p150^{Glued}, DCTN1, has been cloned, and fluorescence *in situ* hybridization was used to map the gene to chromosome 2p13 (Holzbaur and Tokito, 1996). Mutations leading to a form of limb–girdle muscular dystrophy have also been mapped to 2p13 (Bashir *et al.,* 1994); the possibility of a linkage is currently being examined. In mouse, the gene encoding p150^{Glued} has been mapped to chromosome 6 (Vaughan *et al.,* 1996a), a region syntenic with the human localization. The mouse *mnd2* mutation, which results in a phenotype resembling human motor neuron disease, has been mapped to this region of chromosome 6 (Jones *et al.,* 1993); however, a detailed molecular analysis of the p150^{Glued} locus cloned from the mutant mouse strain has revealed no differences in sequence or expression of the polypeptide in comparisons of mutant and wild-type mice (Jang *et al.,* 1997).

The expression of p150^{Glued} in vertebrate tissues has been examined by both Northern and Western blotting. p150^{Glued} is expressed at a low level in all tissues, but its expression is highly enriched in the brain (Gill *et al.,* 1991; Holzbaur *et al.,* 1991; Melloni *et al.,* 1995). These observations have been confirmed by the higher resolution technique of *in situ* hybridization (Melloni *et al.,* 1995). Studies on expression of p150^{Glued} in developing rat indicate that p150^{Glued} is expressed at high levels in neurons from very early in development, particularly in the retinal tissue (Melloni *et al.,* 1995). The temporal and spatial expression pattern of p150^{Glued} in the brain is consistent with its essential role in development as exemplified by the *Gl*^{1} mutation in *Drosophila,* first described by Plough and Ives (1935). *Gl*^{1} is a dominant mutation characterized by pleiotropic developmental defects, especially in the development of the compound eye, resulting in the rough eye phenotype (Plough and Ives, 1935). Flies heterozygous for *Gl*^{1} exhibit gross organizational defects in retinal and optical tissues (Meyerowitz and Kankel, 1978). Homozygous embryos for *Gl*^{1} or null mutants are not viable, indicating that *Glued* is essential for normal development in *Drosophila* (Harte and Kankel, 1982).

Primary sequence analysis of p150^{Glued} predicts an amino-terminal globular head domain followed by extensive heptad repeats, suggesting a coiled coil structure, with a smaller globular domain at the carboxyl terminus

(Fig. 2; Gill *et al.*, 1991; Holzbaur *et al.*, 1991). Initial comparisons of the predicted protein sequences from rat and *Drosophila* revealed the conservation of a highly charged motif near the C terminus of the p150^{Glued} polypeptide. This motif (KKEK) has been shown to be required for the binding of centractin to the p150^{Glued} polypeptide by affinity chromatography and peptide competition studies. In an *in vitro* binding assay, the binding of centractin/Arp1 to an affinity matrix with immobilized recombinant p150^{Glued} was significantly reduced by competition with an exogenous peptide that spans this charged motif (Fig. 2; Waterman-Storer *et al.*, 1995). This motif resembles the sequence in the headpiece domain of villin which has been shown to be required for binding to actin (Friederich *et al.*, 1992).

Molecular characterization of the *Gl*^{1} mutation has shown that it is a result of an insertion of a transposon element at the carboxyl terminus of the polypeptide that results in the synthesis of a truncated *Glued* product (Swaroop *et al.*, 1985; McGrail *et al.*, 1995; Waterman-Storer and Holzbaur, 1996) lacking the KKEK motif required for the *in vitro* association of p150^{Glued} with centractin (Waterman-Storer *et al.*, 1995). Biochemical analysis of dynactin purified from the *Gl*^{1}/+ mutant strain of *Drosophila* by sucrose density gradient centrifugation revealed that in addition to the normal 160-kDa polypeptide which sediments at 19 S, a truncated Glued polypeptide of 145 kDa was found to sediment at ~6.5 S (McGrail *et al.*, 1995). Together, these observations suggest that the C terminus containing the KKEK motif in p150^{Glued} is required for the association of this polypeptide with the centractin filament at the base of the dynactin complex.

2. p150^{Glued} Binds to Microtubules via an Amino-Terminal Cap-Gly Domain

Two other domains have been characterized in the sequence of p150^{Glued} which allow dynactin to associate directly with microtubules and with the microtubule-based motor cytoplasmic dynein. Initial transfection studies in which p150^{Glued} was overexpressed in mammalian cells led to the observation that the polypeptide decorated the microtubule cytoskeleton (Figs. 3c and 3d). Truncation and mapping studies both in transfected cells and in *in vitro* binding assays have shown that amino acids 39–150 are both necessary and sufficient for the binding of p150^{Glued} to microtubules (Waterman-Storer *et al.*, 1995; Tokito *et al.*, 1996). The binding of constructs synthesized *in vitro* to microtubules was quantitated and the dissociation constant was calculated to be 10 μM, suggesting a relatively weak affinity. It is not clear if the formation of a p150^{Glued} dimer within the native dynactin complex would lead to an enhanced affinity for the microtubule.

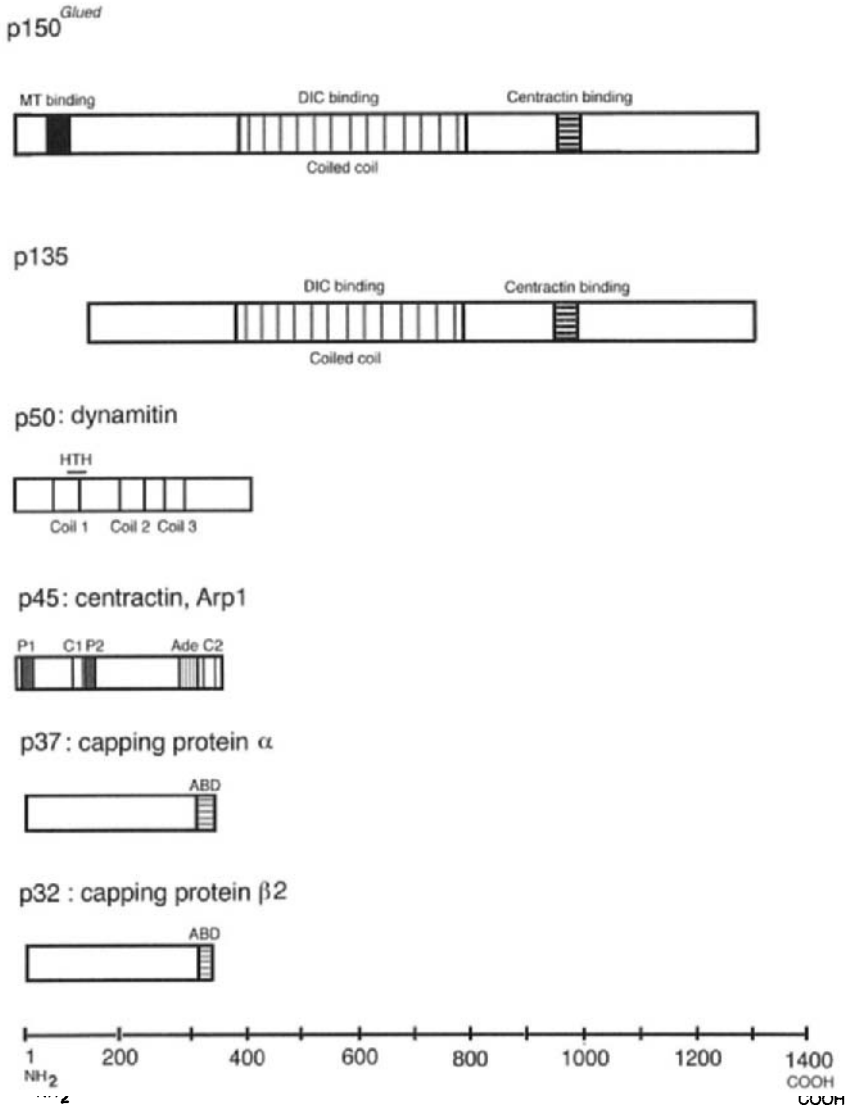

FIG. 2 Block diagrams of predicted domains in the dynactin polypeptides. The p150^{Glued} protein contains an N-terminal Cap-Gly motif that has been characterized as a microtubule-binding domain (Waterman-Storer *et al.*, 1995). The central region of the polypeptide consists of coiled coils that may mediate protein protein interactions. Residues 152 to 811 have been demonstrated to mediate an association of p150^{Glued} with the dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). The C-terminal portion of the protein contains a KKEK motif similar to the charged motif identified in the actin-binding protein villin as necessary for actin binding (Friederich *et al.*, 1992). This motif in p150^{Glued} appears to be responsible for the ability of this polypeptide to bind to centractin *in vitro* (Waterman-Storer *et al.*, 1995) and *in vivo* (McGrail *et al.*, 1995). The p135 polypeptide is encoded by an alternatively spliced varient of p150^{Glued}. This isoform lacks the amino-terminal Cap-Gly

Comparisons of the microtubule-binding domain in p150^{Glued}, referred to as a CAP-GLY motif (Fig. 2; Riehemann and Sorg, 1993), with other sequences in the database have revealed homology with several proteins with diverse sequences, structures, and cellular functions. This group of proteins includes BIK-1, cofactor E, and CLIP-170. The yeast polypeptide BIK1 is required for nuclear fusion, chromosome disjunction, and nuclear segregation during mitosis (Berlin *et al.,* 1990; Trueheart *et al.,* 1987). The polypeptide cofactor E is a required component of the folding pathway for β-tubulin (Tian *et al.,* 1996), and CLIP-170 is a 170-kDa polypeptide which forms homodimers and has been shown to link endosomes to microtubules (Pierre *et al.,* 1992). The binding of CLIP-170 to microtubules has been shown to be mediated by the same microtubule-binding domain as that found in p150^{Glued}. In CLIP-170 this motif is found in two copies as a direct repeat at the amino terminus of the protein. The CLIP-170 holoprotein is thus predicted to bind to microtubules via four copies of this motif, presumably with a relatively high affinity, although this has not yet been assayed. Phosphorylation of CLIP-170 has been shown to induce its dissociation from the microtubule (Rickard and Kreis, 1991).

Evidence from several species clearly indicates that there is alternative splicing of transcripts from the DCTN1 gene encoding p150^{Glued}. While Northern blots of RNA isolated from either rat (Melloni *et al.,* 1995) or mouse (Jang *et al.,* 1997) do not resolve multiple transcripts, three distinct transcripts were seen in an analysis of p150^{Glued} expression in chicken (Gill *et al.,* 1991). Western blots of p150^{Glued} expression in mammalian brain clearly reveal two immunologically related polypeptides of 150 and 135 kDa (Holzbaur *et al.,* 1991; Melloni *et al.,* 1995). A more detailed analysis of the expression of the DCTN1 gene in human neurons has led to the characterization of a second transcript, the expression of which is highly enriched in neuronal cells. Comparisons

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microtubule-binding motif (Tokito *et al.,* 1996). The p50 subunit referred to as dynamitin is characterized by three coiled coil domains and an HTH domain (Echeverri *et al.,* 1996) that has been identified in many prokaryotic transcription factors. The predicted linear protein structure of centractin includes five sequence motifs that together form the "actin fold" which binds nucleotide (Bork *et al.,* 1992; Kabsch and Holmes, 1995). Modeling studies show that the structure of centractin resembles other members of the "actin-fold family." Domains shown are phosphate 1 (amino acids 10–33), connect 1 (134–153), phosphate 2 (155–175), adenosine (295–326), and connect 2 (333–357). Capping proteins (p37 and p32) include actin-binding motifs that are located at the C termini of the polypeptides. While isoforms of capping protein differ in the actin-binding region region due to alternative splicing, these variations do not affect their observed affinities for conventional actin (Schafer *et al.,* 1996). Units denoted by scale bar are approximate values.

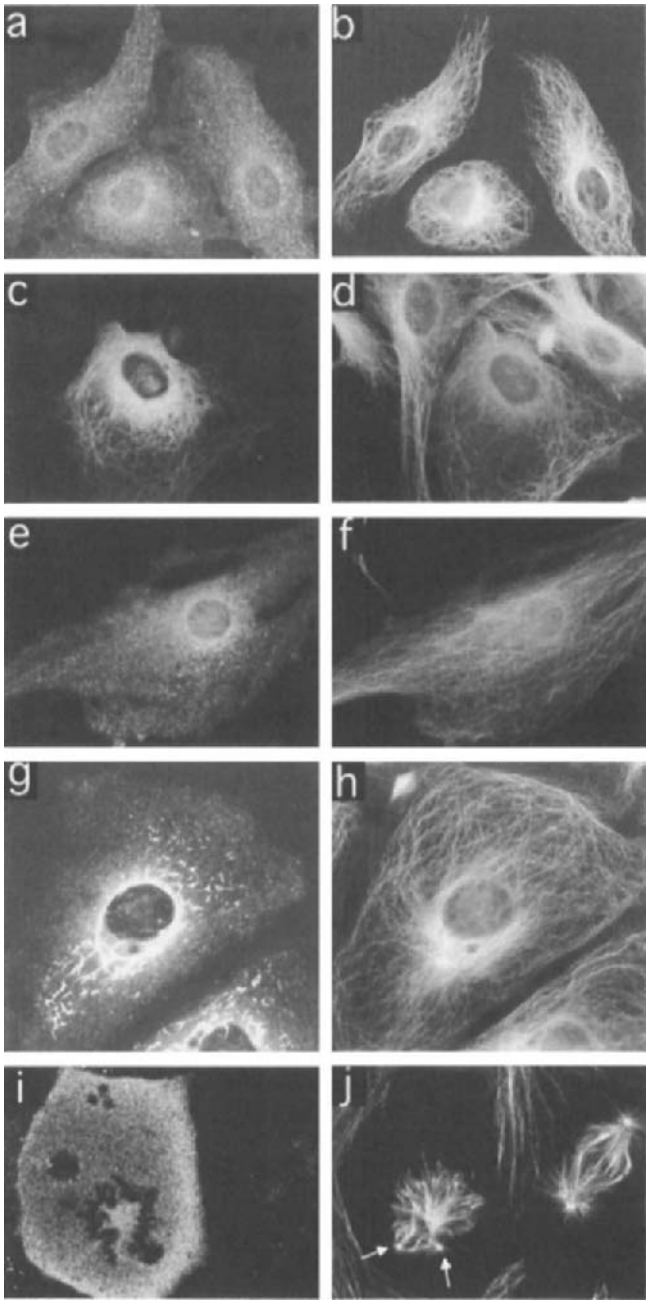

FIG. 3 Overexpression studies in mammalian cells have revealed insight into the function of several subunits of dynactin. In each of the panels to the left, the cells are stained with an antibody raised against one of the dynactin subunits and the same cells are double-stained with antibodies against tubulin as shown in the corresponding panels to the right. (a) Ptk2

of cloned human cDNAs encoding the p150 and p135 isoforms have revealed that the smaller form is an alternatively spliced transcript from the same gene that lacks the coding sequence for the amino-terminal microtubule-binding domain (Tokito *et al.,* 1996). Overexpression of the p135 isoform has revealed that in contrast to p150^{Glued} (Figs. 3c and 3d; Waterman-Storer *et al.,* 1995), the p135 isoform does not decorate microtubules (Figs. 3e and 3f; Tokito *et al.,* 1996). This is consistent with the fact that the shorter isoform is lacking the N-terminal amino acids that have been demonstrated as necessary for p150^{Glued} to associate with microtubules (Waterman-Storer *et al.,* 1995).

The observed stoichiometry of dynactin subunits suggested that the p150 and p135 isoforms coassembled to form the extended arm of dynactin (Paschal *et al.,* 1993; Schafer *et al.,* 1994a). However, immunodepletion studies have shown that p150 and p135 assemble into distinct forms of dynactin which differ in their ability to bind to microtubules (Tokito *et al.,* 1996). While the functional role of either form of dynactin has yet to be completely characterized, it is interesting to note that the p135 isoform appears to be preferentially enriched in the neuronal compartment, corresponding to the slow component of axonal transport (Dillman *et al.,* 1996). Detailed sequence and expression analysis of the human DCTN1 gene is currently underway. Preliminary results indicate that there are multiple isoforms expressed which differ in their inclusion of small exons; it is unlikely that these isoforms will be resolved on standard SDS–PAGE analysis (Tokito *et al.,* 1996; M. K. Tokito and E. L. F. Holzbaur, unpublished data).

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cells stained with anti-centractin antibody. In untransfected cells (a), dynactin subunits colocalize to similar structures in the cell. These diffuse punctate structures are concentrated around the perinuclear and centrosomal regions of the cell. The punctate nature of the staining is indicative of a vesicular association. (b) The same cells stained with anti-tubulin antibodies. (c and d) Overexpression of the p150^{Glued} component reveals that it (c) colocalizes with microtubules (d) (Waterman-Storer *et al.,* 1995; Tokito *et al.,* 1996). (e and f) The p135 isoform that lacks the microtubule-binding domain found in p150^{Glued} and upon overexpression localizes to vesicular structures (e) but does not associate with microtubules (f) (Tokito *et al.,* 1996). (g and h) Overexpression of centractin (g) in mammalian cells results in the formation of filaments that do not colocalize with microtubules (h) but do colocalize with Golgi-specific spectrin and disrupt the Golgi apparatus (Holleran *et al.,* 1996). (i and j) Overexpression of dynamitin (i) results in abberant assembly of the mitotic spindle (j) (Echeverri *et al.,* 1996). (Figs. 3a, 3b, and 3g–j are all reproduced from the *Journal of Cell Biology* **132,** 617–633, 1996, and **135,** 1815–1830, 1996, with permission from the Rockefeller University Press; Figs. 3c–f are reproduced from *Molecular Biology of the Cell* **7,** 1167–1180, 1996, with permission of the American Society for Cell Biology.

3. p150^{Glued} Binds to Cytoplasmic Dynein

The p150^{Glued} subunit of dynactin has also been shown to mediate the binding of dynactin to the microtubule-based motor cytoplasmic dynein. While studies in *Drosophila* and the filamentous fungi *N. crassa* suggested a genetic interaction between cytoplasmic dynein and dynactin, the nature of this interaction was not clear. In *Drosophila,* extragenic suppressors and enhancers of the Gl^1 mutation were mapped to the cytoplasmic dynein heavy chain (McGrail *et al.,* 1995). In *Neurospora,* mutations in either the p150^{Glued} or the Arp1 subunits of dynactin yield a ropy phenotype (described below), which is indistinguishable from the phenotype caused by mutations in the heavy chain of cytoplasmic dynein (Plamann *et al.,* 1994; Robb *et al.,* 1995; Tinsley *et al.,* 1996). In yeast, mutations in Arp1 resulted in a phenotype of inefficient nuclear translocation into the bud which closely resembled the phenotype observed in dynein mutants (Clark and Meyer, 1994; Muhua *et al.,* 1994; Li *et al.,* 1993; Eshel *et al.,* 1993).

A direct interaction between dynein and dynactin mediated by the p150^{Glued} component has been biochemically demonstrated, both by affinity chromatography (Karki and Holzbaur, 1995) and by blot overlay technique (Vaughan and Vallee, 1995). The site of interaction has been localized between amino acids 200 and 811 in the amino-terminal half of p150^{Glued} (Fig. 2; Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), and the first 123 residues of the dynein intermediate chain. As dynactin and dynein may be isolated either individually (at 20 S) or as a single complex with a sedimentation coefficient of ~30 S (Kini *et al.,* 1996), the binding of these two proteins appears to be regulated in the cell. While phosphorylation has been proposed to regulate this interaction, there is no direct evidence as yet. In motility studies in squid axoplasm (Waterman-Storer *et al.,* 1997) and in *Xenopus* extract (Steffen *et al.,* 1997), it has been demonstrated that antibodies specific for either p150^{Glued} or the dynein intermediate chain can block the dynein–dynactin interaction and also block the transport of organelles along microtubules. Therefore, it appears that the direct binding of dynactin to dynein, mediated by the p150^{Glued} subunit, is essential for dynein-mediated transport of membranous vesicles.

4. p150^{Glued} Is Phosphorylated *in Vivo*

Cellular labeling studies have indicated that p150^{Glued} is exclusively phosphorylated on serine residues (Farshori and Holzbaur, 1997). In cultured cells treated with effectors of either protein kinase A or protein kinase C, increased phosphorylation of p150^{Glued} is paralleled by an observed increase in the overall extent of organelle motility. In a mechanism analogous to that observed for CLIP-170 (Rickard and Kreis, 1991), phosphorylation

may regulate the association of dynactin with the microtubule, and this in turn may regulate vesicle motility.

Phosphorylation may also affect the affinity of the binding of p150^{Glued} to cytoplasmic dynein. The serine/threonine kinase CKII has recently been demonstrated to bind to a cytoplasmic dynein intermediate chain affinity column, in an interaction independent of the binding of dynactin to dynein. CKII has also been shown to phosphorylate both cytoplasmic dynein and the p150^{Glued} subunit of dynactin (Karki *et al.,* 1997). As CKII has been shown to bind stably to several of its substrates, these observations suggest that cytoplasmic dynein may be an *in vivo* target for this kinase. While CKII is predominantly found in the nucleus, the kinase has also been localized to the centrosome (Krek *et al.,* 1992) and to the spindle apparatus of dividing cells (Krek *et al.,* 1992; Yu *et al.,* 1991). CKII has been implicated as a regulatory kinase in both cell division and neurite extension. As cytoplasmic dynein has also been implicated in these processes, there may be a specific role for this kinase in regulating dynein and dynactin through the direct phosphorylation of either of these proteins.

In filamentous fungi, genes encoding subunits of both dynein and dynactin have been identified in genetic screens for suppressors of a mutation in the serine/threonine kinase cot-1 (Plamann *et al.,* 1994). It is possible that either dynein or dynactin is a substrate for this kinase *in vitro.*

B. p62—Cloning and Characterization

Immunoelectron microscopy has localized a 62-kDa polypeptide to one end of the centractin filament which forms the base of the dynactin complex (Schafer *et al.,* 1994a). While the sequence of p62 has not yet been reported, preliminary data suggest that the sequence is novel and unrelated to other proteins in the database. Based on the localization of the polypeptide within the dynactin complex, it has been suggested that p62 may promote nucleation of the centractin filament or act as a pointed-end capper (Schafer *et al.,* 1994a). Complete sequence analysis may reveal further insights into the function of this polypeptide within the dynactin complex.

C. p50—Dynamitin

1. Molecular Characterization of p50

p50, or dynamitin, is the second most abundant subunit in the dynactin complex, with an estimated stoichiometry of 4 to 5 moles of p50 per mole of dynactin (Paschal *et al.,* 1993; Schafer *et al.,* 1994a). Sequencing of cDNA

clones from rat and human revealed that p50 is a novel protein, with no previously described homologs, and that it is highly conserved, as the rat and human sequences are 96% identical (Echeverri *et al.,* 1996). Mapping experiments suggest that there is a single gene for p50 (Vaughan *et al.,* 1996a), and Northern blots indicate a single major mRNA species of 1.7 kb (Echeverri *et al.,* 1996). Immunoblots probed with an anti-p50 monoclonal antibody have indicated that this polypeptide is ubiquitously expressed in all tissues examined. A single immunoreactive band was generally observed, although there appeared to be minor differences in the size of the polypeptide in heart, skeletal muscle, and spleen (Echeverri *et al.,* 1996).

Analysis of the primary sequence of p50 predicts that the protein is highly α-helical in nature. Three domains of ~30 residues each have been identified in the center of the polypeptide which have a high probability of forming α-helical coiled coil interactions (Echeverri *et al.,* 1996); however, it has not yet been determined whether the protein self-associates to form oligomers.

While database searches with the dynamitin sequence revealed no clearly related proteins, Echeverri *et al.* (1996) have noted that the predicted structure of the polypeptide is similar to the predicted protein structure for the product of the *JNM1* gene from *Saccharomyces cerevisiae,* characterized by McMillan and Tatchell (1994). Sequence analysis of the predicted 44-kDa product of the *JNM1* gene suggests that the protein is characterized by three coiled coil domains, as predicted for the dynamitin polypeptide. *JNM1* has been shown to be required for nuclear migration and mitotic spindle orientation in yeast (McMillan and Tatchell, 1994). As described below, the phenotype of JNM1 mutants in this gene closely resembles that observed for mutants in which the expression of the cytoplasmic dynein heavy chain is disrupted, and therefore it is possible that JNM1 is the yeast homolog of mammalian dynamitin. This hypothesis was further substantiated by coimmunoprecipitation of JNM1p (Kahana *et al.,* 1996) with the yeast homologues Nip100 (p150^{Glued}) and Act5p (centractin).

2. Overexpression of p50 Disrupts Mitotic Spindle Assembly

Transfection studies with p50 have provided an intriguing view into the cellular role of dynactin. In interphase cells, the overexpressed polypeptide was found to localize diffusely throughout the cytoplasm. Preliminary reports have indicated that this overexpression may cause the redistribution of endosomes and lysosomes from the perinuclear region of the cell toward the cell periphery (Burkhardt *et al.,* 1995), and the disruption of ER to Golgi trafficking (Presley *et al.,* 1996). The disruption in the Golgi due to overexpression of p50 is reminiscent of the effects on the Golgi upon microtubule depolymerization. These observations lend support to the idea

that dynactin may be involved in the positioning of Golgi in the perinuclear region, as has previously been suggested for cytoplasmic dynein (Courthesy-Theulaz *et al.*, 1992).

Analysis of the transfected cell population indicated that an abnormally high percentage of the transfected cells were found to be mitotic (9%, compared to 2% of control cells; Echeverri *et al.*, 1996). These transfected cells showed a prometaphase-like configuration of their chromosomes. Significant aberrations were detected in the morphology of the mitotic spindles in these cells. The spindles were asymmetric and lacked astral microtubules (Figure 3j; Echeverri *et al.*, 1996).

Domain analysis of the p50 polypeptide is currently underway and suggests that the first 105 residues of dynamitin are necessary but not sufficient to induce both prometaphase arrest and dispersal of the Golgi apparatus (Echeverri and Vallee, 1996). Biochemical analysis of the transfected cells revealed that the overexpressed polypeptide was not associated with other dynactin subunits. Remarkably, while only 10% of the cells were transfected and overexpressing p50, biochemical analysis of the cytosolic extracts from these cultures revealed a complete dissociation of $p150^{Glued}$ from dynactin. This observation suggests that the presence of excess p50 in the extract was sufficient to disrupt dynactin in the cytosol from the untransfected cells as well (90% of total cells). Together, these data suggest that p50 is critical to the assembly of dynactin and that intact dynactin is essential in mitotic spindle assembly.

D. p45—Centractin (Arp1)

1. Centractin Is a Member of the Actin-Related Protein Family

The 45-subunit of dynactin is a member of a growing family of actin-related proteins. The actin-related proteins were first identified based on the similarity of their sequences to those of conventional actins. The conventional actin sequences share greater than 75% identity across species and tissue type; however, the sequence identity of the actin-related protein family to actin is lower and ranges from 28 to 62%. Members of this unconventional actin family have been classified based on the overall degree of sequence similarity, as well as sequence insertions and deletions relative to actin (Mullins *et al.*, 1996; Frankel and Mooseker, 1996; Schroer *et al.*, 1994). The 45-kDa subunit of dynactin, centractin, has been defined as a member of the Arp1 class, which shares ~50% sequence identity with conventional actin (Schroer *et al.*, 1994).

To date, over 20 actin-related proteins have been identified in organisms ranging from *S. pombe* to human. Many of the actin-related proteins have been identified by serendipity in an attempt to clone out other genes of interest. The actin-related proteins appear to have important but very distinct roles in the cell. Briefly, Arp2 and Arp3 have been identified as subunits of a large complex of 7 different and apparently novel proteins (Machesky *et al.,* 1994; Kelleher *et al.,* 1995; Mullins *et al.,* 1997). This complex has been shown to bundle actin filaments (Mullins *et al.,* 1997). A similar complex isolated from platelet extracts may have a role in *Listeria monocytogenes* movement in the cytoplasm of mammalian cells (Welch *et al.,* 1997). A nuclear localization has been observed for the actin-related proteins Arp13E (Frankel *et al.,* 1994) and Act3p (Harata *et al.,* 1995; Weber *et al.,* 1995), suggesting that these proteins may be involved in chromatin packaging (Weber *et al.,* 1995; Frankel *et al.,* 1994). Arp53D, expressed in *Drosophila* testis, is currently the Arp most closely related to actin based on sequence comparisons (Fyrberg *et al.,* 1994). The extended family of actin-related proteins has recently been reviewed by Mullins *et al.* (1996) and Frankel and Mooseker (1996).

Overall, the sequence similarity of the actin-related proteins is greatest in the regions that have been shown to form the nucleotide binding cleft, or "actin fold," in conventional actin (Kabsch and Holmes, 1995). The sequences have been shown to vary more extensively in the regions which are predicted to be externally exposed (Lees-Miller *et al.,* 1992; Clark and Meyer, 1992; Kelleher *et al.,* 1995). These observations raise the question of whether these actin-related proteins are capable of forming polymers, *in vivo.* At present, only centractin, an Arp1, has been shown to form polymers in the cell (Schafer *et al.,* 1994a; Holleran *et al.,* 1996).

Initial studies with antibodies to centractin revealed that in vertebrate cells the protein localizes to the centrosomal region of the cell and thus the protein was named for centrosomal-associated actin (Clark and Meyer, 1992). In addition, centractin is concentrated around the perinuclear region and is diffuse throughout the cytoplasm (Clark and Meyer, 1992; Holleran *et al.,* 1996). The punctate nature of the staining is indicative of vesicle association. The localization of centractin is similar to that of the other dynactin complex members. Over time, as the cells become more confluent and more stably attached to the coverslips, the accumulation and localization of centractin at the centrosome increase (Holleran and Holzbaur, unpublished results). However, extensive treatment of cultured cells with nocodozole (16 h) causes both the disruption of cellular microtubules and the loss of dynactin staining at the centrosome, suggesting that dynactin is not an integral centrosomal component and instead is most likely associated with the minus ends of microtubules (Paschal *et al.,* 1993).

2. Molecular Characterization of Arp1

Centractin was initially cloned from both human and canine cDNA libraries (Clark and Meyer, 1992; Lees-Miller *et al.,* 1992). The predicted protein sequences of the human and canine cDNAs are identical, each encoding a protein of 376 residues. Arp1 sequence homologues have also been identified in *S. cerevisiae* (Clark and Meyer, 1994; Muhua *et al.,* 1994), *N. crassa* (Plamann *et al.,* 1994), *Pneumocystis carinii* (Christopher *et al.,* 1995), *C. elegans* (Shrimankar *et al.,* 1994), and *Drosophila melanogaster* (Fyrberg *et al.,* 1994). While it is not yet clear whether each of these sequence homologues is a true functional homologue, in several species genetic studies have indicated that these proteins may be involved in similar or identical cellular processes.

Northern blots of HeLa cell mRNA provided evidence of isoform diversity in the expression of centractin. Clark *et al.* (1994) have described three distinct bands of 3.0, 2.0, and 1.3 kb which hybridized to a centractin cDNA probe. These are referred to as α-centractin, β-centractin, and γ-centractin, respectively. α-centractin and β-centractin are present at similar ratios in all mRNA tissue samples except in lung tissue where β-centractin mRNA is less abundant. α-centractin and β-centractin mRNAs are more abundant in pancreas and brain tissues than in liver or lung tissues. While γ-centractin has not been fully characterized, γ-centractin is found only in heart muscle, skeletal muscle, and lung tissue. It is possible that γ-centractin mRNA is derived from a separate gene or, alternatively, that γ-centractin could be a splice variant of α-centractin.

In vertebrate tissues centractin appears to be ubiquitously expressed (Clark and Meyer, 1992), but at a level significantly below actin. In HeLa cells centractin has been estimated to comprise 0.006% of total cellular protein, and in yeast less than 0.00022% of total protein, or less than 0.07% of actin expression levels (Muhua *et al.,* 1994).

The predicted sequence of the β-centractin protein is 91% identical to that of the α-centractin protein with the differences in sequence distributed throughout the entire molecule. Both α-centractin and β-centractin proteins comigrate on sucrose gradients at 20S and are present within the dynactin complex at a ratio between 10:1 and 20:1 (α:β). Two-dimensional gels can separate the α- and β-centractin polypeptides (42.6 vs 42.3 kDa) from one another because of a slight difference in isoelectric points (Clark *et al.,* 1994).

In *S. cerevisiae,* a centractin homologue, Act5p, has been identified based on 53% sequence identity to vertebrate centractin (Clark and Meyer, 1994; Muhua *et al.,* 1994). In yeast, Southern blot analysis reveals that there is only one gene (Clark and Meyer, 1994; Muhua *et al.,* 1994). The *N. crassa* gene *ro-4* was originally identified as a suppressor of a mutation in the *cot-1* gene which encodes a serine/threonine kinase. Sequence analysis of *ro-4*

revealed a predicted protein product that is 65% identical to vertebrate centractin (Plamman *et al.,* 1994). An Arp1 gene has also been identified in *C. elegans. C. elegans* Arp1 is 67% identical to vertebrate centractin. The gene has been mapped chromosome II and the probe also showed weak hybridization to the right end of chromosome III (Shrimankar *et al.,* 1994).

In *D. melanogaster* the centractin homologue ARP87C was identified using PCR amplification with degenerate oligonucleotides to the ATP-binding cleft of conventional actin (Fyrberg *et al.,* 1994). The predicted protein is 79% identical to vertebrate centractin. In a study of the organization and function of indirect flight muscle by Schoenenberger *et al.* (1995), transgenic *Drosophila* were used to study the effect of ectopically expressed vertebrate α-cardiac actin, β-cytoplasmic actin, and centractin in null mutants for the IFM-specific actin Act88F. While α-cardiac actin (28 residues different from Act88F) transgenic flies displayed well-ordered sarcomeres, β-actin (only 4 residues that diverge from Act88F) and centractin transgenic flies had disordered myofibrils and sarcomeres and were unable to fly. While these results certainly do not rule out the possibility that centractin could play a role in sarcomere organization, these observations do indicate that centractin and actin have distinct cellular roles.

In *P. carinii,* a centractin homologue was identified by PCR amplification of cDNA templates generated from isolated mRNA using degenerate oligonucleotides to conventional actin (Christopher *et al.,* 1995). The protein is recognized by affinity-purified antibodies to *S. cerevisiae* Act5p and canine centractin, is composed of 385 amino acids, and has a predicted molecular weight slightly larger than 45 kDa. The predicted peptide sequence is 52% identical to Act5p form *S. cerevisiae,* 69% identical to vertebrate centractin, and 74% identical to *ro-4* from *N. crassa.* Interestingly, the Arp1 sequence homologue in *P. carinii* is most concentrated in nuclear extracts.

3. Structural Analysis of Centractin

Centractin forms a short filament at the base of the dynactin complex (Schafer *et al.,* 1994a). It has been estimated that this filament contains 8 to 13 centractin monomers (Schafer *et al.,* 1994a). Capping protein (discussed below) and the p62 component of dynactin have been localized to opposite ends of the centractin filament (Schafer *et al.,* 1994a).

Primary sequence alignment of centractin with conventional actin reveals that the two polypeptides are 53% identical. Much of this identity is found in the nucleotide and cation-binding regions in actin. These five regions, distributed throughout the length of the protein, consist of an adenine-binding domain, two connecting regions, and two phosphate-binding re-

gions that fold to form an ATP-binding core structure referred to as the "actin fold" (Bork *et al.,* 1992; Kabsch and Holmes, 1995).

We have used molecular modeling to compute the predicted three-dimensional model of monomeric centractin (Fig. 4). FASTA was used to align the sequence (Z14978 and J00805) and coordinates of conventional actin (PDB Accession No. 1ATN; Kabsch *et al.,* 1990) with those of centractin (X82206). A backbone structure for centractin was first generated by assigning the known coordinates from the actin structure to residues conserved in the sequence of centractin. Coordinates that generated the most minimal structural perturbations were then assigned to the divergent residues. The insertions in centractin (glutamine 98 and glutamine 240) relative to actin were modeled to produce the most favorable loop conformations. The deletion of four residues in centractin relative to actin (near aspartic acid 231) was modeled by removing the first turn of the α-helix between subdomains 3 and 4. Finally, the model structure was refined using the Discover energy minimization program to minimize side chain bumping in the model.

The resulting structural model predicts that the overall topology of centractin closely resembles that of actin. Sixteen of seventeen residues that bind nucleotide and cation in actin are conserved in centractin. In addition, molecular modeling reveals that residues 137–144 in actin are conserved in centractin both in sequence and in structure. In actin, these residues form an α-helical hinge that allows the two halves of the molecule to rotate by twisting at valine 339 and open like scissors at alanine 331 to allow nucleotide binding and release (Schutt *et al.,* 1993; Tirion and ben-Avraham, 1993).

4. Nucleotide Binding

Structural studies on actin have led to the identification of a core structure for nucleotide binding, referred to as an actin fold (Bork *et al.,* 1992; Kabsch and Holmes, 1995). This structural motif has been identified in a number of ATP-binding proteins such as FtsA, MreB, and StbA (Bork *et al.,* 1992; Sanchez *et al.,* 1994). Sequence comparisons and structural predictions suggest that centractin also shares this structural motif and therefore binds nucleotide. To test this possibility, Melki *et al.* (1993) found that centractin synthesized in an *in vitro* transcription/translation system can bind to nucleotide immobilized on an agarose matrix. However, in these studies no differences were detected in the affinity of centractin for either GTP or ATP. We have performed site-directed mutagenesis of residues predicted to be crucial for nucleotide binding in centractin, and the results support the hypothesis that centractin binds nucleotide and that this binding is

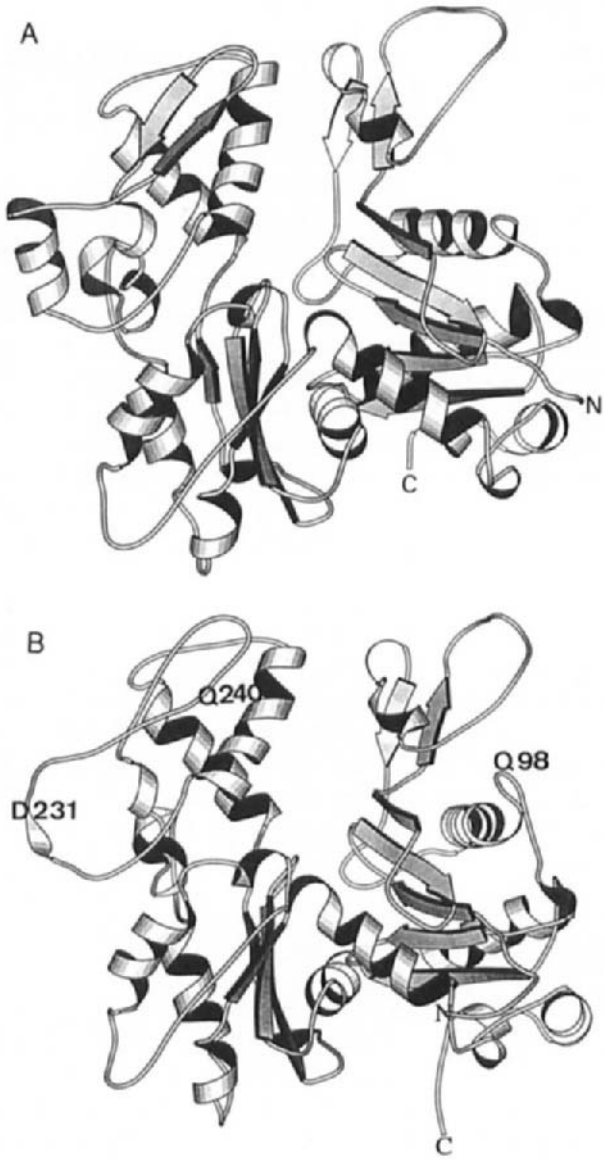

FIG. 4 Predicted three-dimensional structure of centractin (Arp1). (A) Ribbon diagram of the crystal structure of conventional actin (PDB Accession No. 1ATN; Kabsch *et al.*, 1990). (B) Ribbon diagram of the predicted three-dimensional structure of centractin (Arp1). The overall topology of the predicted structure of centractin is very similar to that of conventional actin. Centractin contains a deletion relative to actin in the first turn of the α-helix between subdomains 3 and 4 near aspartic acid 231. Glutamine 98 and glutamine 240 are insertions relative to actin. We performed three-dimensional modeling of centractin by FASTA alignment of the sequence of centractin with that of the structural coordinates determined for conventional actin using the Insight II v. 2.0 (Biosym Corp.) and Discover energy minimization programs.

dependent on residues conserved between actin and centractin (Holleran and Holzbaur, manuscript in preparation).

5. Pharmacology

Cytochalasin D and phalloidin are two cytotoxins which have been demonstrated to perturb the dynamics of actin polymerization. However, centractin appears to differ significantly from actin in its interaction with these reagents. Holleran *et al.* (1996) have demonstrated that phalloidin does not bind to the novel centractin filaments that are formed in mammalian cells that are overexpressing centractin. These centractin filaments in transfected cells are not disrupted by treatment with cytochalasin D under conditions which lead to the complete depolymerization of the actin cytoskeleton (Holleran *et al.*, 1996).

6. Protein–Protein Interactions

Sequence comparisons and structural modeling studies suggest that the core structures of actin and centractin are similar but that the surface residues of the folded polypeptides differ significantly. Therefore, some, but not all, of the proteins which interact with conventional actin may also bind to centractin. Likely candidate proteins that may interact with the actin-related protein include actin itself, chaperonins, and conventional actin-binding proteins such as capping protein (a stoichiometric component of dynactin), spectrin-related proteins, and unconventional myosins. Some of these proteins have already been tested for an interaction with centractin and are discussed below.

Melki *et al.* (1993) have demonstrated that like actin and α- and β-tubulin (Gao *et al.*, 1992, 1993; Frydman *et al.*, 1992; Yaffe *et al.*, 1992), centractin requires a multisubunit heteromeric chaperonin to fold correctly. The unfolded centractin polypeptide forms a complex with chaperonin; release of the folded polypeptide from the complex requires MgATP. Centractin competes with β-actin in binding to cytoplasmic chaperonin, indicating that both of these cytoskeletal proteins require the same chaperonin for proper folding and function (Melki *et al.*, 1993).

The interaction of actin monomers to form an actin polymer has been well characterized, and the amino acid residues involved in this association are well understood. These residues are partially conserved in centractin and raise the intriguing possibility that actin and centractin can copolymerize. Initial *in vitro* studies suggested such a direct interaction. Melki *et al.* (1993) synthesized centractin in an *in vitro* transcription/translation system and found that this trace amount of protein cosedimented with F-actin. Also, it was initially reported that actin was a stoichiometric component

of dynactin. To address the possibility of copolymerization, centractin was overexpressed in mammalian cells, resulting in the production of novel filaments throughout the cytoplasm. Actin was not incorporated into these filaments, and centractin was not incorporated into actin filaments. Even under conditions in which the filamentous actin cytoskeleton is completely disrupted and allowed to reform in the presence of excess centractin, there was no incorporation of centractin into the reforming F-actin (Holleran *et al.,* 1996). Immunoprecipitations of the dynactin complex also revealed that while dynactin subunits and other actin-binding proteins coprecipitate (described below), actin does not form a high-affinity association with the dynactin complex (Holleran *et al.,* 1996).

Further analysis of cells transfected with centractin revealed an association between centractin and spectrin. Antibodies to the Golgi-specific isoform of spectrin (Beck *et al.,* 1994) colocalized with the centractin filaments. This association between centractin and spectrin is also seen in cytosolic fractions from rat brain (Holleran *et al.,* 1996) and from membrane preparations from epithelial cells (Fath *et al.,* 1996). Adducin, a protein that promotes an association between spectrin and actin (Li and Bennett, 1996), was also found to coimmunoprecipitate with antibodies to dynactin (Holleran *et al.,* 1996). These data suggest a model in which dynactin may associate with intracellular organelles through an association of the 37-nm centractin filament with organelle-associated spectrin (Fig. 5A; Section IV).

Mullins *et al.* (1996) have hypothesized that several other actin-binding proteins may bind to centractin. These include thymosin β4/actobindin, profilin, annexin, villin, gelsolin, myosin, tropomyosin, and α-actinin. Thymosin β4 and profilin associate primarily with G-actin, and while it is possible that they may also bind to a single centractin monomer, centractin exists almost exclusively as a member of the dynactin complex, and thus in a polymeric form (Paschal *et al.,* 1993). It is possible that during biogenesis of the dynactin complex, centractin may interact with monomeric actin-binding proteins such as these. There are at present no data suggesting an interaction of other actin-binding proteins with dynactin; however, the existence of multiple isoforms of many actin-binding proteins such as members of the unconventional myosin superfamily (Cheney *et al.,* 1993) raises the possibility of interactions of such proteins with centractin.

E. p37 and p32—Capping Protein

1. Identification

Both protein microsequencing and antibody cross-reactivity have been used to identify the 37- and 32-kDa components of dynactin as the α and β

subunits of capping protein, or CapZ (Schafer *et al.*, 1994a). CapZ was originally isolated from chicken muscle (Casella *et al.*, 1989) and the localization of this protein to the Z line suggested an involvement in the binding to the barbed or plus end of actin filaments (Casella *et al.*, 1989). Since its discovery in muscle, CapZ has been identified in nonmuscle cells (Caldwell *et al.*, 1989; Cooper *et al.*, 1991). Capping protein nucleates actin filament assembly from monomeric actin and therefore increases the rate of actin polymerization (Casella *et al.*, 1986, 1987; Caldwell *et al.*, 1989).

Schafer *et al.* (1994a) performed microsequencing of the 32-kDa polypeptide which copurifies with dynactin and discovered that 8 of 11 residues matched the reported sequence for the $\beta 2$ isoform of capping protein. Antibodies to capping protein recognized both the 37- and the 32-kDa dynactin subunits (Schafer *et al.*, 1994a). In structural studies, antibodies to capping protein have been localized to a single end of the centractin filament of native dynactin (Schafer *et al.*, 1994a), suggesting that, by analogy with actin, capping protein may only associate with one end of the centractin polymer.

2. Characterization of Isoforms

Isoform diversity has been observed for both the α and the β subunits of capping protein (Schafer *et al.*, 1994b). Two isoforms of the α subunit have been resolved by two-dimensional gel electrophoresis of chicken tissue extracts; a third putative α isoform has also been detected (Schafer *et al.*, 1996). cDNAs encoding $\alpha 1$ and $\alpha 2$ isoforms have been characterized from several species, including human (Barron-Casella *et al.*, 1995, and unpublished data from the Cooper laboratory; Accession Nos. P52907 and P47755). Analysis of dynactin purified from chick brain by two-dimensional gel electrophoresis followed by immunoblotting has suggested that there are two distinct α isoforms in the dynactin complex (Schafer *et al.*, 1994a,b)

In chick, it has been shown that β subunit of capping protein is alternatively spliced to form two different isoforms, $\beta 1$ and $\beta 2$. These two isoforms differ in the C-terminal portion of the molecule that has been identified as the actin-binding domain (Hug *et al.*, 1992; Casella and Torres, 1994). The $\beta 1$ and $\beta 2$ isoforms have different tissue distributions. $\beta 1$ is the predominant form expressed in muscle, and $\beta 2$ is the predominant isoform expressed in nonmuscle cells (Schafer *et al.*, 1994b). The intracellular distributions of the two isoforms were compared in cardiac muscle cells. The $\beta 1$ isoform was localized to the Z discs of myofibrils, while the $\beta 2$ isoform of capping protein is concentrated at intercalated discs, cell–cell junction sites, and sites of attachment of the myofibril to the sarcolemma (Schafer *et al.*, 1994b). Interestingly, only the $\beta 2$ isoform has been found as a subunit of the dynactin complex (Schafer *et al.*, 1994a). In fibroblasts, the $\beta 2$ isoform

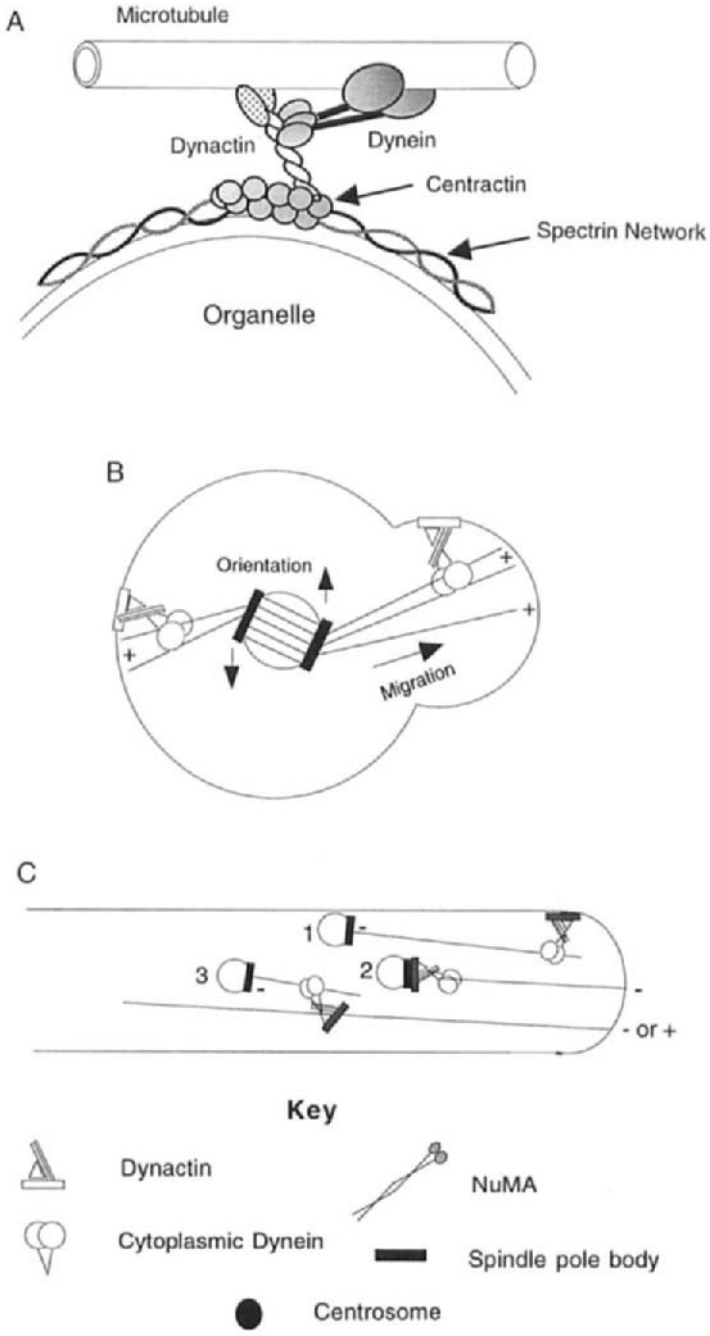
A
Microtubule
Dynactin
Dynein
Centractin
Spectrin Network
Organelle
B
Orientation
Migration
+
+
+
C
1
2
3
-
-
- or +
Key
Dynactin
NuMA
Cytoplasmic Dynein
Spindle pole body
Centrosome

FIG. 5

FIG. 5

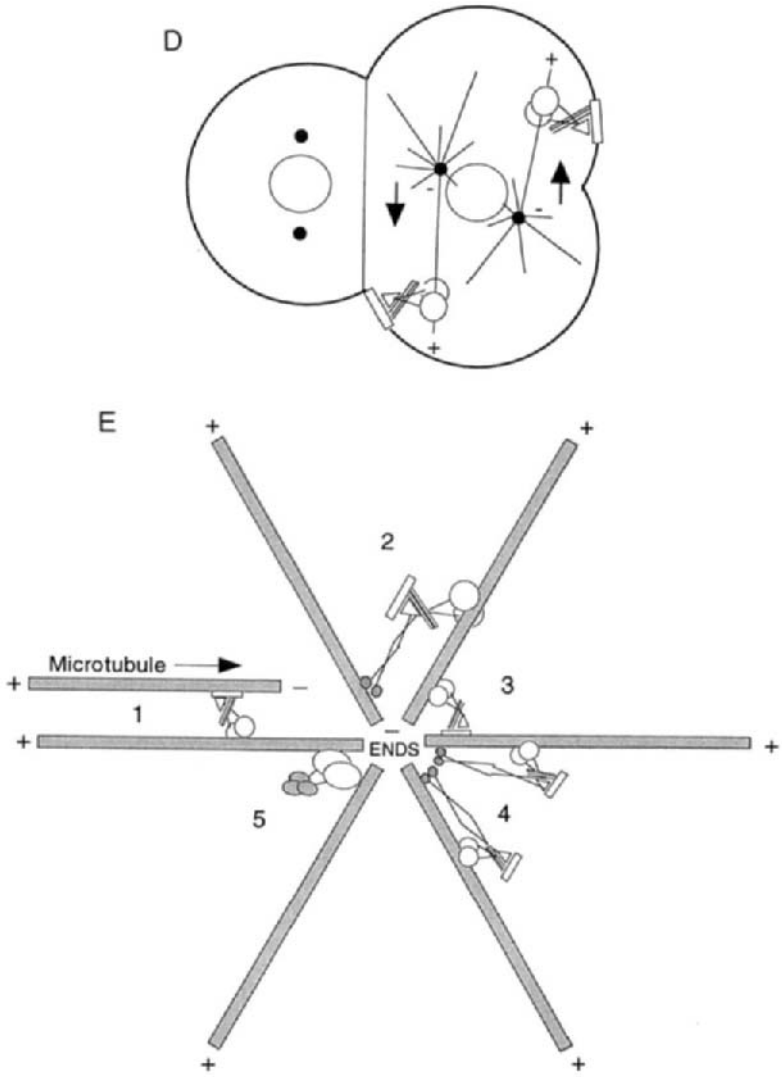

FIG. 5 Models depicting possible roles of dynein/dynactin. (A) Vesicle transport. Numerous data have implicated dynactin's involvement in vesicle transport; this model is a composite of several studies. The morphology of dynactin with the centractin minifilament and the appearance of p150^{Glued} sidearm projection is based on immunoelectron studies by Schafer *et al.* (1994a). The interaction between dynein and dynactin was demonstrated biochemically (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). The microtubule-binding activity of p150^{Glued} was studied both *in vitro* and in transient cellular transfections (Waterman-Storer *et al.*, 1995). Holleran *et al.* (1996) have demonstrated that centractin (Arp1) associates with the spectrin network, providing a possible linking mechanism for dynactin with the membrane. Direct support for the requirement of dynein/dynactin interaction for vesicle motility comes from experiments done on the extruded squid axoplasm (Waterman-Storer *et al.*, 1997). Cross-reactive rabbit polyclonal anti-p150^{Glued} antibody (UP235) was observed to block bidirectional

FIG. 5 (Continued) microtubule-based motility in the extruded axoplasm. Biochemical analysis showed that this inhibition of motility was due to disruption of dynein/dynactin interaction by UP235. (B) Spindle orientation and nuclear migration in budding yeast. In yeast, dynein and dynactin mutants exhibit similar phenotypes. In *Saccharomyces cerevisiae* the spindle has to be oriented properly at the neck before migration can proceed. This model is based on Eshel *et al.* (1993), suggesting that while the elongation of antiparallel spindle microtubules may be due to a kinesin-like motor, the orientation of the spindle, and subsequent migration of the nucleus, is probably due to cytoplasmic dynein which is anchored to the cell cortex of the mother cell and the bud. In this model dynein attached to the bud cortex "walks" toward the minus ends of astral microtubules, which in effect pulls the elongated spindle to the bud while the dynein attached to the mother cell keeps the other half of the spindle in the mother cell. We suggest that this anchoring mechanism may involve the Arp1 polypeptide of dynactin (Holleran *et al.*, 1996). See also Li *et al.* (1993), McMillan and Tatchell (1994), Muhua *et al.* (1994), and Morris *et al.* (1995). The attachment model is consistent with the observation that the mutations in the dynein heavy chain or dynactin components such as Jnm1p and Arp1 have misaligned spindles and defects in nuclear migration. (C) Nuclear migration in filamentous fungi. Genetic analysis has revealed that dynactin and dynein are involved in similar processes. Mutations in the dynein heavy chain or subunits of dynactin in the filamentous fungi *Aspergillus nidulans* (Osmani *et al.*, 1990; Xiang *et al.*, 1994, 1995) and *Neurospora crassa* (Plamann *et al.*, 1994; Tinsley *et al.*, 1996; Robb *et al.*, 1995) exhibit uneven distribution of nuclei. The model depicted here is modified from Morris *et al.* 1995). Three possible modes of nuclear migration are shown by the three numbered nuclei. Nucleus 1 is being pulled by dynein which is anchored near the tip of the mycelium by dynactin. Here the "walking" of cortically attached dynein on a microtubule connected to the spindle pole body causes the nucleus to move closer to the hyphal tip. Nucleus 2 is directly attached to the motile dynein/dynactin complex which is simply moving toward the minus end of a microtubule. This model requires that the minus ends of microtubules be oriented toward the hyphal tip. Nucleus 3 is moving in a fashion identical to nucleus 1 except that the dynein/dynactin complex is not at the tip of the hypha; instead, it is attached to a microtubule by $p150^{Glued}$ which has its own microtubule-binding site. (D) Centrosome pair rotation in *C. elegans.* Dynactin subunits may be involved in centrosome pair rotation during development. The model shown here is adapted from Waddle *et al.* (1994). In early *C. elegans* embryogenesis, the first cleavage gives rise to two cells; the anterior daughter (AB) is larger than the posterior (P1). Subsequent cleavage patterns of these two daughter cells are different. The AB lineage adopts synchronous, equal divisions at right angles to the previous cleavage whereas the P1 lineage adopts unequal, asynchronous divisions on the same axis. The latter pattern depends on the rotation of duplicated centrosome pair just before division (Hyman and White, 1987). Waddle *et al.* (1994) suggest that the centrosome pair rotation is dependent on actin and microtubule polymerization and that actin and CapZ transiently accumulate at cortical sites where a microtubule may be attached. This attachment may be provided by dynactin, via a link between the centractin minifilament and a cortical spectrin network (Holleran *et al.*, 1996). Cytoplasmic dynein tethered to dynactin at the cortex "walks" along the astral microtubules, which in effect rotates the centrosome pair (indicated by arrows) to take an anterior–posterior position. (E) Organization of astral microtubules. It has been demonstrated that cytoplasmic dynein is required for organization of microtubules into mitotic asters as well as for efficient localization of NuMA to the poles (Gaglio *et al.*, 1996). This model depicts how NuMA, cytoplasmic dynein, and dynactin may organize astral microtubules (modified from Gaglio *et al.*, 1996). Scheme 1 shows how dynein which is attached to an astral microtubule may slide another microtubule toward the organization center with the minus end end of the sliding microtubule pointing toward the center. Scheme 2 shows how the dynein/dynactin/NuMA complex may function together to cross-link adjacent microtubules. Here dynactin is depicted as a common interactor of dynein and NuMA. In scheme 3, the dynein/dynactin complex alone is depicted

was observed to be distributed in a punctate pattern throughout the cytoplasm, consistent with the localization of other dynactin subunits (Schafer *et al.,* 1994b).

The recent demonstration that actin is not a subunit of dynactin (Holleran *et al.,* 1996) suggests that capping protein binds directly to centractin. The studies of Muhua *et al.* (1994) provide genetic evidence for an interaction between capping protein and Arp1. They have determined that in yeast a *cap1/act5* double mutant has a more severe phenotype than a single act5 mutant with respect to nuclear migration. However, direct binding of capping protein to centractin has not yet been demonstrated.

F. p27 and p22

The smaller subunits of dynactin have yet to be completely characterized. p27 and p24 (now known as p22) were reported to associate at a stoichiometry of 1 mole of each subunit per 1 mole of dynactin complex (Schafer *et al.,* 1994a). However, further biochemical characterization of a 27-kDa polypeptide which copurifies with dynactin by affinity chromatography revealed that this is a protein distinct from dynactin. Karki *et al.* (1997) have identified the 27-kDa polypeptide as the β subunit of casein kinase II. Both the α and the β subunits of casein kinase II bind to the intermediate chain of cytoplasmic dynein, in an interaction which is independent of the interaction between dynein and dynactin. This kinase phosphorylates both dynein and dynactin *in vitro;* the physiological relevance of this association is currently being explored. It still remains to be determined if there is a stoichiometrically associated 27-kDa subunit of dynactin.

The smallest subunit of dynactin (originally referred to as p24) has now been identified both biochemically and molecularly as a 22-kDa polypep-

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as cross-linking adjacent microtubules. While the directionality of the dynein motor assures localization of the dynein/dynactin complex at the minus end of the microtubule, the independent microtubule-binding activity of dynactin helps to cross-link microtubules. Scheme 4 is different from scheme 2 only in the sense that self-assembly of NuMA to form a tetramer, for example, assures cross-linking of adjacent microtubules via dynein. This model is simplified to indicate that NuMA is associated with dynactin which in turn binds to dynein (Gaglio *et al.,* 1996; Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Scheme 5 is less likely where dynein alone is depicted to cross-link minus ends of adjacent microtubules. Schemes 3 and 5 both require the condition that dynein and dynactin acquire mitosis-specific behavior. Thus, NuMA may play a role in conferring a specific function to the dynein/dynactin complex during mitosis. As a result, we favor models 2 and 4 where dynein, dynactin, and NuMA are involved in the aster organization.

tide. p22 is currently being characterized in several labs. Sequence analysis has revealed that p22 is apparently a novel protein with with no clearly defined sequence motifs. The polypeptide is ubiquitiously expressed in vertebrate tissues, and Northern and Western blot analyses indicate no evidence for isoform diversity of this subunit (Karki and Holzbaur, submitted for publication). Immuno-EM studies on dynactin did not resolve the localization of this polypeptide within the complex, and biochemical analysis has not yet provided information on intramolecular associations.

III. Mutational Analyses

A. *D. melanogaster*

The first mutant in a dynactin subunit was identified in 1935, by Plough and Ives, as a dominant allele which resulted in aberrant formation of the compound eye and optic lobe of *Drosophila.* Molecular characterization of the Gl^1 mutation demonstrated that it is a result of an insertion of a transposon element at the carboxyl terminus of the polypeptide that results in a truncated *Glued* product (Swaroop *et al.,* 1985). This truncation results in the loss of a highly conserved motif at the carboxyl terminus of the polypeptide that has been shown to be required for the association of $p150^{Glued}$ with centractin (Waterman-Storer *et al.,* 1995). The molecular basis for the rough eye phenotype in Gl^1 flies is likely to be due to the formation of nonfunctional dynactin because the *Glued* polypeptide cannot interact with centractin. Biochemical analysis of dynactin isolated from Gl^1/+ heads using sucrose density gradient centrifugation revealed that while the wild-type 160-kDa polypeptide sedimented as a component of the dynactin complex at 19S, a truncated mutant 145-kDa polypeptide sedimented at ~6.5S (McGrail *et al.,* 1995). These results indicate that the mutant form of the polypeptide cannot assemble with other dynactin polypeptides. As the mutant $p150^{Glued}$ is predicted to retain a functional binding site for cytoplasmic dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), the truncated polypeptide may compete for dynein binding. This competition would explain the dominant negative nature of the observed Gl^1 phenotype, potentially disrupting retrograde transport of growth factors during neurite extension and therefore altering the normal patterns of neuronal outgrowth.

Genetic studies in *Drosophila* clearly support the biochemical identification of a direct interaction between cytoplasmic dynein and dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Dynactin and dynein colocalize throughout *Drosophila* oogenesis, and the localization of *Glued*

during oogenesis has been shown to depend on cytoplasmic dynein (McGrail *et al.,* 1995). Gepner *et al.* (1996) have generated a series of mutations in the cytoplasmic dynein heavy chain gene. The strongest mutant alleles of *Dhc* result in lethality at the larval stage. It appears that the mutants survive through most of embryogenesis due to maternal contributions. Some mutations in cytoplasmic dynein also display a rough eye phenotype similar to that observed in Gl^1 mutants (McGrail *et al.,* 1995). Further, the ability of certain dynein heavy chain mutations to act as enhancers or suppressors of the rough eye phenotype of Gl^1 lends support to the idea of their functional interaction (McGrail *et al.,* 1995). Mosaic analyses have indicated that the dynein mutants operated as cell lethals, as was observed for the null mutations in *Glued* (Harte and Kankel, 1982).

Mutations in the 8-kDa dynein light chain have also been characterized in *Drosophila.* Null mutations in *cut up* (*ctp*) survive the larval stage; lethality occurs late in pupal development. *ctp1* mutants display mild defects in grooming behavior and are female sterile (Phillis *et al.,* 1996). Detailed analysis of the mutant flies demonstrated significant differences in axonal trajectories within the central nervous system, suggesting that dynein is required for normal axonal pathfinding. However, despite the altered trajectories observed in the mutants, the axons appeared to reach their normal regions of termination. While mutations in *Glued, dhc,* and *ctp* all result in developmental and/or neurological abnormalities consistent with the involvement of these proteins in similar cell functions, the differences observed in the mutant phenotypes may reflect differential requirements for these polypeptides which are not yet understood.

B. *S. cerevisiae*

In *S. cerevisiae,* mutations have been analyzed in genes encoding presumed homologs of the dynamitin, centractin, and capping protein subunits of dynactin. The yeast *JNM1* gene is thought to encode the yeast homolog of the vertebrate dynamitin polypeptide (Echeverri *et al.,* 1996). Analysis of *JNM1* null mutants has shown that this gene is required for nuclear migration into the bud (McMillan and Tatchell, 1994). While *JNM1* is not an essential gene in yeast, null mutants are characterized by an abnormally high frequency of binucleated cells. Defects in spindle orientation were also common in mutant strains.

A putative centractin homologue was identified in *S. cerevisiae* based on sequence homology. The yeast ACT5 gene is predicted to encode a polypeptide which is 53% identical to vertebrate centractin (Muhua *et al.,* 1994; Clark and Meyer, 1994). Deletion of the ACT5 gene resulted in defects in nuclear migration and spindle apparatus orientation relative to

wild-type yeast. The *act5* null mutant strains were characterized by increased numbers of anucleate and binucleate cells compared to wild type. The severity of the phenotype was enhanced by growth at a lower temperature.

The phenotypes observed in both the *jnm1* and the *act5* mutants closely resemble the phenotypes described for mutants in the cytoplasmic dynein heavy chain (Li *et al.,* 1993; Eshel *et al.,* 1993). *dhc, jnm1,* and *act5* mutants all exhibit misalignment of the spindle apparatus between the mother and the bud. Spindles often remained in the mother cell at an angle to the mother–bud axis with the astral microtubules extending into the bud. In anucleate cells and larger yeast cells, the microtubules were often bundled, elongated, and abnormal in dynein and dynactin mutants.

Null mutants have also been constructed for the yeast *CAP1* and *CAP2* genes, which encode the α and β subunits of capping protein, respectively. Null mutants in either gene are viable and exhibit a similar phenotype. The mutant cells are large, rounded, and show disrupted organization of their actin cytoskeleton (Amatruda *et al.,* 1990; Amatruda and Cooper, 1992). However, the analysis of this phenotype is complicated by the multiple cellular roles of capping protein, which appears to be both a critical regulator of the actin cytoskeleton and a subunit of dynactin.

C. Filamentous Fungi: *N. crassa* and *Aspergillus nidulans*

The role of dynactin in nuclear migration has also been explored in the filamentous fungi *N. crassa.* Ropy mutants, *ro,* were originally identified as partial suppressors of defects in hyphal growth caused by a mutation in the *cot-1* gene, which encodes a member of the cAMP-dependent protein kinase family (Yarden *et al.,* 1992). *ro* mutations are characterized by curled hyphal growth and uneven distribution of nuclei along the hyphae. *ro-4* has been shown to encode an actin-related protein which is the probable homolog of vertebrate centractin (Plamann *et al.,* 1994; Robb *et al.,* 1995), and *ro-3* encodes p150^{Glued} (Tinsley *et al.,* 1996). The phenotype of these mutations in dynactin subunits is indistinguishable from mutations in *ro-1,* which encodes the cytoplasmic dynein heavy chain gene. Defective nuclear migration due to defects in cytoplasmic dynein has also been characterized in the filamentous fungi *A. nidulans* (Xiang *et al.,* 1994). The genetic evidence for an association between dynein and dynactin strongly supports the biochemical evidence of a direct interaction between these two complexes.

D. *C. elegans*

The role of Arp1 has been investigated in *C. elegans.* During development of the *C. elegans* embryo, an unequal distribution of specific cytoplasmic

and maternally supplied determinants occurs at the two-cell (AB and P1) stage of the embryo. The two daughter cells, AB and P1, each inherit a centrosome from the P0 cell. In the P1 cell, the centrosomes migrate and then rotate 90° to form a longitudinal mitotic spindle along the anterior–posterior axis of the embryo (Allen and Kropf, 1992; Hyman and White, 1987; Palmer *et al.,* 1992). The proper rotation of the centrosomal pair in the P1 daughter cell, the correct orientation of the cleavage furrow, and the proper spindle orientation and localization are all crucial in the process of cytoplasmic partitioning (Strome, 1993). Rotation of the centrosome is sensitive to inhibitors of both actin and microtubule polymerization, indicating that the process is dependent on an intact cytoskeleton (Hyman and White, 1987). In addition, the centrosome movement may depend upon a cortical region near the cell midbody which may function as an attachment site for astral microtubules to act in centrosome rotation (Hyman, 1989; Hyman and White, 1987). It has also been suggested that dynactin may be necessary for the rotation of the P1 cell centrosome (Shrimankar *et al.,* 1994).

In fixed *C. elegans* embryos, Arp1 protein has been localized to midbodies which are known as cell division remnants (Shrimankar *et al.,* 1994). At the completion of cytokinesis Arp1 localizes to the cell division remnant between AB and P1 daughter cells. Localization of centractin through the cell cycle is also coincident with the movement of vesicle structures that move from the EMS and P2 surfaces to the interior. Since Arp1 has spatial and temporal localization similar to that of the vesicular structures, it has been hypothesized that the cell division remnant and Arp1 may also be closely associated with these structures (Shrimankar *et al.,* 1994). Capping protein (discussed below) and actin also transiently accumulate at the cell division remnant site (Waddle *et al.,* 1994). The rotation of the centrosomes during development may depend on a minus-end-directed motor localized at the cortex of the cell. It is possible that dynactin may interact with such a motor to provide tension on astral microtubles (Waddle *et al.,* 1994). It is also possible that a plus-end motor such as kinesin creates a force on the microtubule closer to the centrosome (Waddle *et al.,* 1994). Thus, in a process perhaps similar or analogous to nuclear migration (as in fungi) dynactin may play a role in centrosome migration and spindle alignment in *C. elegans.*

E. Mouse

Studies on the role of dynein and dynactin in higher eukaryotes using transgenic and knockout technologies may be hampered due to the essential nature of these proteins. Harada *et al.* (1996) have made an initial report

of the knockout of the cytoplasmic dynein heavy chain in mice by gene targeting. While heterozygote mice were found to be phenotypically normal and fertile, thus indicating haplosufficiency, no homozygous mice could be recovered. This observation suggests that the null phenotype is embryonic lethal. At present, no data are available on mice with knockouts in dynactin genes, but based on the observations with dynein these might be predicted to be embryonic lethal as well.

F. Transfection Studies

Due to difficulties with transgenic approaches, most studies on the role of dynactin in higher eukaryotes have focused on the use of transient transfection assays to overexpress either wild-type or mutant constructs of dynactin polypeptides. Studies have examined the cellular effects of overexpressing the p150^{Glued}, p135, centractin, and p50 (dynamitin) subunits (Waterman-Storer *et al.,* 1995; Tokito *et al.,* 1996; Holleran *et al.,* 1996; Echeverri *et al.,* 1996).

Overexpression of p150^{Glued} in cultured mammalian cells led to the identification and characterization of the microtubule-binding site within this polypeptide but did not reveal any significant cellular defects (Figs. 3c and 3d). Although cells were transfected with constructs mimicking the carboxyl-terminal truncation of the *Gl*^{1} mutation, a dominant negative phenotype was not recapitulated in culture (Waterman-Storer *et al.,* 1995). While this might reflect differences between the vertebrate and insect polypeptides, it is more likely that over the course of the transient transfection experiment the defect was not sufficiently severe to result in a lethal phenotype. In this study, a thorough survey for a mitotic defect was not performed. Additional studies of the p135 isoform, which is lacking the amino-terminal residues necessary for the binding of p150^{Glued} to microtubules, indicate that this polypeptide localizes to punctate vesicular structures and does not colocalize with microtubules upon overexpression (Figs. 3e and 3f; Tokito *et al.,* 1996).

A mitotic defect was detected in the analysis of transfected cells overexpressing dynamitin (Figs. 3i and 3j; Echeverri *et al.,* 1996). Nine percent of cells transfected with p50 were found to have a prometaphase-like cellular configuration, in comparison to 4% of untransfected or β-gal-transfected control cells. More tellingly, in prometaphase cells overexpressing p50, significant aberrations were detected in the morphology of the mitotic spindle. Spindles were asymmetric in size and shape, and the orientation of the two halves of the spindle appeared to be independent of each other.

In contrast to the mitotic phenotype resulting from overexpression of p50, transfections with centractin have provided insight into the role of dynactin in organelle transport along microtubules. Overexpression of cen-

tractin led to the formation of novel filaments, which either were distributed throughout the cytoplasm or were perinuclear (Figs. 3g and 3h; Holleran *et al.,* 1996). In both cases, these filaments colocalized with markers for the Golgi apparatus, suggesting an association with this organelle. The identification of an association between centractin and spectrin both by analysis of transfected cells and by coimmunoprecipitation experiments has led to the model shown in Fig. 5A and described below.

IV. Models for the Function of Dynactin in Intracellular Transport

A. Vesicle Transport

Both *in vitro* and *in vivo* studies have suggested dynactin's essential role in vesicular transport. Gill *et al.* (1991) demonstrated that while immunodepletion of dynactin from dynein preparations did not affect the rate or extent of microtubule gliding, this depletion blocked the motility of vesicles along microtubules. In studies in extruded squid axoplasm, Waterman-Storer *et al.* (1997) have observed that antibodies which block the dynein–dynactin interaction block the motility of organelles along microtubules, implying a direct role of dynactin in axonal transport. Both of these studies suggest that dynactin is essential in order to couple the motor protein dynein to drive vesicular motility (Fig. 5A).

The mechanism by which dynactin activates or potentiates dynein's motor function remains to be explored. The simplest possibility is that dynactin serves as a vesicle-bound receptor for dynein, so that in the absence of dynactin dynein can no longer productively associate with the membrane. While it has been shown that cytoplasmic dynein may directly associate with phospholipid vesicles (Lacey and Haimo, 1994), it is unclear if this *in vitro* association is physiologically relevant or of sufficient strength to maintain the link during force production. Instead, the results of Holleran *et al.* (1996) on the association of dynactin with spectrin suggest that dynactin associates with the organelle via an association with a membrane-bound cytoskeleton composed of spectrin, adducin, and possibly ankyrin. The direct binding of dynein to dynactin would then allow for a specific association of the motor with the organelle.

It has also been proposed that dynactin may serve as a tethering mechanism, maintaining the close association of the organelle with the microtubule (Waterman-Storer *et al.,* 1995). The pathway for ATP hydrolysis and force production for the cytoplasmic form of dynein has not yet been investigated in any detail, but these studies have been performed on the

axonemal enzyme. Kinetic studies have determined that the slow step of the axonemal dynein ATPase cycle is ADP release (Holzbaur and Johnson, 1989a) and that this step is activated by microtubules (Holzbaur and Johnson, 1989b). At physiological levels of ATP, each head will be detached from the microtubule for up to 80% of its duty cycle. While both cytoplasmic and axonemal dyneins are multiheaded motors, with two to three sites for force generation per complex, studies on the axonemal enzyme have revealed no cooperative behavior between these sites (Johnson *et al.,* 1983; Shimizu and Johnson, 1983; Holzbaur and Johnson, 1989b). This kinetic observation is consistent with structural observations in which the connection of the heads to the common base is via relatively extended stalks (Johnson and Wall, 1983; Vallee *et al.,* 1988). Therefore, under physiological ATP concentrations the probability of both independent heads becoming detached from the microtubule is relatively high. These results are in contrast to observations on kinesin, as kinetic analysis of this motor has suggested an alternating site or hand-over-hand model, in which a single head detaches from the microtubule at any one time (Gilbert *et al.,* 1995; Hackney, 1995; Vale *et al.,* 1996). In consequence, kinesin is a highly processive motor, making long runs along a microtubule without detaching.

While a lack of cooperativity between the two dynein heads may be easily tolerated within the dense array of the axoneme, where a detached dynein head will see a high "local concentration" of binding sites along the microtubule of the adjacent doublet, the situation appears to differ significantly within the cell. If dynein is the only link holding a vesicle to a microtubule, and if both heads become detached simultaneously, then the organelle may diffuse away from the microtubule prior to rebinding of either head. This would make for an inefficient transport system, with low levels of processivity. There are three possibilities which may prevent this situation. One, the kinetics of cytoplasmic dynein may differ significantly from those of axonemal dynein, and this must be analyzed in detail. Two, dynein may be concentrated in patches on the organelle surface, and thus the binding and release of multiple unsynchronized motors may prevent diffusion. Or third, there may be a tethering mechanism which allows for a weak association of the vesicle with the microtubule. Dynactin is an excellent candidate for this type of tether, as it binds both to dynein and to the microtubule (Fig. 5A; Karki and Holzbaur, 1995; Vaughan and Vallee, 1995; Waterman-Storer *et al.,* 1995). The direct association of dynactin with the microtubule appears to be rather weak, characterized by a dissociation constant of 10 μM (Waterman-Storer *et al.,* 1995). This may be strong enough to prevent diffusion if both dynein heads become detached, yet weak enough to be "dragged along" during the force generation step. In support of this hypothesis is the observation that when vesicle motility in squid extracts is blocked with an antibody which blocks the

dynein–dynactin interaction, the vesicles are observed to become tethered to the microtubules of the axoplasm, rather than freely diffusing (Waterman-Storer *et al.,* 1997).

An alternate hypothesis for the role of dynactin in activating dynein suggests a key role in "loading" in that dynactin brings dynein, the microtubule, and the organelle together as a ternary complex which can then undergo motility (Vaughan *et al.,* 1996b). In this model, the binding of dynactin to the microtubule is predicted to be regulated, potentially by phosphorylation. There is some evidence to support this model also, as the microtubule-binding site within p150^{Glued} is homologous to a microtubule-binding site within CLIP-170 which is regulated by phosphorylation (Rickard and Kreis, 1991). While it has been demonstrated that p150^{Glued} is a phosphoprotein (Farshori and Holzbaur, 1997), there are as yet no data indicating that phosphorylation effectively regulates the association of dynactin with microtubules.

B. Nuclear Migration

Studies in yeast and filamentous fungi have clearly revealed a role for both dynein (Eshel *et al.,* 1993; Li *et al.,* 1993; Plamann *et al.,* 1994; Xiang *et al.,* 1994) and dynactin (Clark and Meyer, 1994; Muhua *et al.,* 1994; Plamann *et al.,* 1994) in nuclear migration. Models for this process in yeast suggest that dynein is generating a pulling force along astral microtubules (Eshel *et al.,* 1993) and predict that dynein is bound to the cellular cortex and reaches into the cell to grab and pull on astral microtubules emanating from the nuclear envelope-associated spindle pole body (Figs. 5B and 5C). Cortical association of dynein has been detected in yeast by Yeh *et al.* (1995), who found that this association is independent of dynein activity. This model raises the question of the mechanism of cortical attachment of the dynein motor, which may perhaps be addressed by the recent discovery of an association between dynactin and spectrin (Holleran *et al.,* 1996). It is has been suggested that dynactin may associate with cortical spectrin in a cell-cycle-dependent manner (Holleran *et al.,* 1996). Thus, dynactin could then serve as a binding platform for cytoplasmic dynein on the cortical face of the plasma membrane.

In a potentially similar process, dynactin may play a role in centrosome migration and spindle alignment in the developing embryo, as suggested by recent work in *C. elegans.* Studies in this organism have revealed the localization of dynactin to cortical patches, or cell division remnant sites (Shrimankar *et al.,* 1994). The rotation of the centrosomes and therefore spindle assemblies observed during development may be dependent on dynactin localized at the cortex of the cell, which will allow dynein to bind and to generate tension on the astral microtubules (Fig. 5D).

C. Mitosis

Recent studies have also clearly established an essential role for dynactin in the assembly of the mitotic spindle (Fig. 5E; Gaglio *et al.,* 1996; Merdes *et al.,* 1996). Immunodepletion studies have revealed an association between NuMA, cytoplasmic dynein, and dynactin. NuMA is localized to the nucleus in interphase cells but becomes concentrated at the ends of the mitotic spindle during mitosis; this localization to the spindle poles is required for the accurate assembly of the spindle (Compton and Cleveland, 1994; Cleveland, 1995). When extracts were depleted of either cytoplasmic dynein or dynactin, microtubules failed to organize into mitotic asters, in contrast to control extracts. These experiments suggest that dynactin may function to facilitate or stabilize the interaction of cytoplasmic dynein with microtubule asters (Gaglio *et al.,* 1996), a role which is similar to the proposed role of dynactin in vesicular motility (Holleran *et al.,* 1996). Alternatively, the assembly of the spindle and the organization of parallel microtubule arrays may require microtubule-cross-linking activity, and it is interesting to note that dynein, dynactin (Waterman-Storer *et al.,* 1995), and NuMA (Merdes *et al.,* 1996) each have microtubule-binding sites. As overexpression of the p50 subunit of dynactin was observed to result in the aberrant formation of mitotic spindles in transfected cells, a role for dynactin in spindle assembly appears to be well established.

Less clear is a potential role for dynactin in later stages of mitosis. Several studies have localized both dynein and dynactin to kinetochores (Steuer *et al.,* 1990; Echeverri *et al.,* 1996), and Echeverri *et al.* (1996) describe a prometaphase arrest in their p50 transfection experiments. However, there is no direct evidence for dynein's function in chromosome-to-pole movements. Instead, studies in yeast have suggested a role for dynein in anaphase which involves generation of an outward force by dynein pulling on the spindle poles (Saunders *et al.,* 1995; Yeh *et al.,* 1995). In yeast, however, dynein's role in mitosis is not essential. Current work in higher eukaryotes may reveal additional nuances of function (Robinson *et al.,* 1996).

V. Concluding Remarks

The analysis of the structure and function of dynactin has continuously provided unanticipated surprises to investigators in the field. For example, the discovery of a novel actin-related protein that acts as a structural component of dynactin and the recent demonstrations of the association of dynactin with both spectrin and NuMA are exciting new directions to pursue. It is relatively easy to predict that work over the next few years will result

in further unexpected insights into the cellular role of this large and complex molecule. The molecular characterization of each of the dynactin subunits will soon be complete, and the insights gained from these studies will be critical. Extended analysis of dynactin mutants will provide more information on the pleiotropic cellular roles of dynactin and dynein. The accelerating pace of research in this area will soon lead to answers to many of the questions that still remain regarding intracellular functions of the dynein–dynactin complex. These answers will also lead to the longer term goal of assessing the role of dynactin within the cell and the organism as a whole.

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Dual Mechanisms of Apoptosis Induction by Cytotoxic Lymphocytes

Joseph A. Trapani
The John Connell Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, 3084, Australia

Cytotoxic T lymphocytes and natural killer cells together comprise the means by which the immune system detects and rids higher organisms of virus-infected or transformed cells. Although differing considerably in the way they detect foreign or mutated antigens, these cells utilize highly analogous mechanisms for inducing target cell death. Both types of effector lymphocytes utilize two principal contact-dependent cytolytic mechanisms. The first of these, the granule exocytosis mechanism, depends on the synergy of a calcium-dependent pore-forming protein, perforin, and a battery of proteases (granzymes), and it results in penetration by effector molecules into the target cell cytoplasm and nucleus. The second, which requires binding of FasL (CD95L) on the effector cell with trimeric Fas (CD95) molecules on receptive target cells, is calcium independent and functions by generating a death signal at the inner leaflet of the target cell membrane. Exciting recent developments have indicated that both cytolytic mechanisms impinge on an endogenous signaling pathway that is strongly conserved in species as diverse as helminths and humans and dictates the death or survival of all cells.

KEY WORDS: Cytotoxic lymphocyte, Perforin, Granzyme, Caspase, Apoptosis, Fas.

I. Introduction

Higher organisms such as mammals have evolved sophisticated immune systems that serve to discriminate between the host and the external environment. For such animals, the greatest challenge to survival is the constant battle to detect and destroy foreign pathogens, a function subserved by the immune system. Many micro-organisms including most bacteria and parasites set up extracellular foci of infection that are amenable to detection and destruction by nonspecific antimicrobial factors such as lysozyme, by

direct phagocytosis or, if necessary, by a specific immune response induced in the humoral arm of the immune system. Following binding of specific immunoglobulin, effector mechanisms such as complement activation and phagocytosis are activated, clearing the pathogen, and memory lymphocytes are retained in case of reexposure to the same or a closely related organism expressing the same antigenic determinants.

By contrast, intracellular pathogens such as viruses and some bacteria require rapid internalization for their pathogenicity, as they must utilize host mechanisms for replication, protein synthesis, and their other metabolic requirements. This life cycle also provides a haven inside host cells, enabling evasion by antibody and less specific "innate" antimicrobial mechanisms. Such organisms thus gain time to replicate and spread to uninfected cells at the opportune moment. As discussed below, all cells have inbuilt mechanisms for detecting a broad variety of cellular damage including that induced by intracellular pathogens. Clearly, it is desirable that infected or otherwise damaged cells (particularly those carrying defective genes) do not replicate, and generic mechanisms are rapidly recruited to block movement of such cells into or through the cell cycle. More severely damaged cells may be induced to actively trigger their own demise, a process called "programmed cell death." Morphologically and functionally, these cells die by apoptosis, a rapid process of cellular collapse and involution quite distinct from toxin-induced necrosis. Cell death in such circumstances is often described as "altruistic" and, in the context of an intracellular infection, is advantageous to the host as it ensures that spread of the pathogen to normal tissues is stopped, at the cost of one host cell. It is not surprising that through the course of evolution, many viruses have devised ways of subverting or at least delaying the normal progression to apoptosis. In such an event, intracellular infections present to the immune system a potentially serious problem of recognition and elimination of a cryptic and perhaps even quiescent (noncytopathic) parasite.

Over the past 15 years, the nature of the receptors used by T lymphocytes to specifically detect foreign (and some self) antigens has become elucidated. Detailed analyses of T cell receptor structure and function, the signaling pathways which follow receptor engagement in the context of self histcompatibility molecules, T cell activation, cytokine cascades, and recruitment of cytotoxic effector cells are beyond the scope of the present review. Suffice it to say that T cells are generated that possess exquisitely specific receptors capable of detecting cells that are judged to be animical and therefore targeted for destruction. These T cells proliferate in response to antigen and costimulatory signals provided by antigen presenting cells and acquire potent lytic function to eliminate unwanted cells.

This chapter will explore the ways in which cytolytic lymphocytes (CL) induce death in these targeted cells. The lymphocyte compartment contains

two different types of effector cell that utilize very similar, if not identical, cytolytic mechanisms. Cytotoxic T lymphocytes (CTL) are generally $CD3^+CD8^+$ T cells, express cytocidal molecules in an inducible manner following exposure to antigen, and can affect cytotoxicity following T cell receptor ligation. By comparison, natural killer (NK) cells recognize foreign antigen by the use of a very different array of facilitatory and inhibitory receptors, which have become the topic of intense recent investigation (Karre, 1997; Lanier *et al.,* 1997; Selvakumar *et al.,* 1997). NK cells appear to utilize basically the same mechanisms as CTL for inducing cytolysis; however, NK cells have a broader spectrum of antigenic specificity. In keeping with their role in the immediate response to virus infection, NK cells express lytic mediators and important cytokines such as interferon-γ (IFNγ) in a constitutive manner, thus either eradicating or at least confining the infection until a specific T cell response can be mounted. Unless otherwise stated, the remarks below concerning cytolytic pathways can be taken as applying to both classes of CL.

II. Mechanisms of Cytotoxic-Lymphocyte-Mediated Cell Death

A. Overview

1. Granule Exocytosis

The process of unraveling the pathways involved in CL-mediated cytolysis has followed a stuttering course since the first demonstration that *in vitro* cytolytic activity was present in transplanted kidney tissue (Govaerts, 1960). The first pathway to be substantially defined, the induced-secretion or granule-exocytosis mechanism, was principally based on ultrastructural studies which reflected "state of the art" technology of the day. Although the general hypothesis of secretion-induced cell death first formulated during the 1970s and early 1980s has proven remarkably resilient (Young and Cohn, 1986), the molecular basis for the pre- and postmortem changes observed in the target cell resisted elucidation for many years and is still the subject of debate. Apparently inconsistent and even contradictory findings added substantially to the controversy: with the benefit of hindsight, we now see that many of these controversies arose because of a hitherto unsuspected second, granule-independent mechanism mediated by Fas ligation. Finally, advances in molecular technologies have provided indisputible evidence for two independent cytolytic pathways whose relative biological

significance is now under intense study using genetic techniques, particularly gene knockout mice.

The idea that lysis could be brought about by exocytosis of specific mediators elaborated from CL was initially proposed by the laboratory of Berke. This group demonstrated that transient direct contact between the effector and the target (conjugate formation) preceded death of the target cell, while the CTL was refractory to death and indeed could recycle to additional target cells (Zagury *et al.*, 1975). Electron microscopy provided some structural evidence to support this concept. First, cytoplasmic granules were noted to become reoriented within the cell close to the site of cell–cell contact in CTL target conjugates (Bykovskaja *et al.*, 1978), and second, discrete lesions were observed by Henkart and his colleagues to appear specifically on the target cell membrane as a result of encountering CTL (Doumarshkin *et al.*, 1980). However, these early reports attracted only scant attention, and it was not until Podack and colleagues confirmed the earlier observations and identified the target membrane lesions as being remarkably similar to those induced by complement that interest grew (Podack and Dennert, 1983). The same investigators demonstrated that a protein which they purified and called perforin was by itself capable of inducing changes in permeability of the target cell membrane and that perforin was present in high concentration within the presynaptic granules. This work embodied the hypothesis that prestored lytic mediators could be released by the CL in a vectorial fashion toward the target cell surface, following antigenic recognition and stabilization of the conjugate by adhesion structures (Dennert and Podack, 1983).

Not only did a large body of evidence supporting the granule exocytosis hypothesis rapid accumulate (detailed below), but the elegant simplicity of the mechanism was inherently appealing. As intuitive as the mechanism appeared, however, several paradoxical observations brought into question the universality and (in some minds) even the underlying validity of this hypothesis (Berke, 1991; Krahenbuhl and Tschopp, 1991). First, some CTL (in particular the peritoneal exudate lymphocytes, PEL) could kill in the apparent absence of calcium ions, which were known to be indispensible for both exocytosis and the pore-forming activity of perforin. Second, some CTL were potent killers yet produced little if any perforin, were essentially nongranulated, and could kill without the need for exocytosis (Dennert *et al.*, 1987; Berke and Rosen, 1988; Berke, 1989; Allbritton *et al.*, 1988; Ostergaard *et al.*, 1987; Ostergaard and Clark, 1989; Trenn *et al.*, 1987). The third issue followed on from the studies of several groups including that of Russell, who demonstrated that target cells frequently died by "internal disintegration" (apoptosis), a mode of death in which nuclear collapse and DNA fragmentation were known to be early and crucial phenomena, but in which cell membrane damage was a later and less

prominent feature (Russell, 1983; see also Schmid *et al.,* 1986). Purified perforin could kill cells by virtue of its potent lytic potential (bringing on a type of necrotic death); however, most investigators were unable to demonstrate the nuclear changes of apoptosis such as DNA fragmentation with perforin alone (Duke *et al.,* 1989). The resolution of these apparent inconsistencies took many years and has involved two broad areas of work which will be considered briefly in turn here, and then in more detail later in this review. The first group of studies centered on the requirement for a synergy of perforin with other granule proteins to bring about apoptosis, as distinct from membrane damage alone. The second body of work involved the demonstration that T cells (particularly the $CD4^+$ Th1 cell) can utilize an alternative but equally potent, granule- and calcium-independent mechanism to bring about rapid target cell apoptosis (Podack, 1995). The observation that CTL kill via the Fas ligand (CD95L)/Fas (CD95) mechanism both solved the conundrum of cytolysis in the absence of perforin and brought CTL-mediated cell death mechanisms into the mainstream of apoptosis research.

2. Perforin and Granzymes Synergize to Induce Target Cell Apoptosis

The debate as to a causal role for perforin in target cell death was finally and unequivocally settled only when data from perforin-deficient gene knockout mice were presented. The initial report of mice deficient in perforin came from Kagi *et al.* (1994a); however, the results since have been independently corroborated by three other groups (Lowin *et al.,* 1994; Kojima *et al.,* 1994; Walsh *et al.,* 1994). Perforin-deficient animals have apparently normal T cell development, with normal numbers of T cells in their peripheral lymphoid organs, normal CD4/CD8 ratios, and no obvious perturbation of T cell cytokine production (Kagi *et al.,* 1994a). The $CD8^+$ T cells were activated normally following infection with lymphocytic choriomeningitis virus (LCMV); however, *in vitro* cytotoxic activity was markedly deficient against LCMV-infected targets when compared to that of the T cells of perforin-expressing littermates. This deficiency in cytolysis was also observed in primary *in vitro* stimulated alloreactive T cells, and to a substantial extent against alloreactive tumor cell lines (Kagi *et al.,* 1994a). NK activity was also virtually abolished in these mice. These studies settled beyond doubt that perforin-mediated cytolysis was the predominant mechanism for protection against virus infection, the principal means for eliminating alloreactive cells, and the dominant mechanism used by NK cells. It incidentally provided a model for studying alternative cytolytic pathways in the absence of the granule exocytosis mechanism (see below).

The intriguing observation that purified perforin alone could not induce an apoptotic morphology strongly hinted that molecules other than perforin were also required for apoptosis induction. Apart from perforin, the most abundant constituents of CTL/NK granules are a family of serine proteases termed "granzymes" (Masson and Tschopp, 1987; Smyth and Trapani, 1995). Granzymes have highly conserved structures and are biochemically and genetically closely related to serine proteases synthesized by mast cells and polymorphs (Smyth and Trapani, 1995; Smyth *et al.,* 1996b). Due to their diverse protease specificities, granzymes have been proposed to participate in many lymphocyte functions (see below). A role for granzymes in CTL-mediated cytolysis had been proposed many years earlier, based on the observations that various protease inhibitors could abrogate cytotoxicity (Chang and Eisen, 1980; see also Hudig *et al.,* 1991). The earlier studies utilized broadly reactive inhibitory compounds, but these have now been superceded by far more specific synthetic oligopeptide isocoumarin or chloromethylketone derivatives such as Boc-ala-ala-**asp**-CH_2Cl, a specific inhibitor of granzyme B, a protease which, unlike other granzymes (or any other known mammalian serine protease), cleaves substrate proteins specifically at key asp residues (Poe *et al.,* 1991). Granzymes do not have apoptosis-inducing or DNA-fragmenting activity when used in isolation *in vitro,* and cells exposed to purified or recombinant granzymes remain viable indefinitely. However, a series of experiments by Henkart and colleagues first showed that granzyme A could induce DNA fragmentation in membrane-disrupted cells (Hayes *et al.,* 1989). Granules isolated from large granular lymphocytes were able to mediate DNA release from ^{125}I-UdR-labeled target cells. Purified perforin could induce only ^{51}Cr release from double-labeled targets, but when it was added back to granules in which perforin had been inactivated, perforin was able to reconstitute both membrane damage and DNA fragmentation (Hayes *et al.,* 1989; Sutton *et al.,* 1997). These findings were corroborated and greatly extended by the group headed by Arnold Greenberg. A DNA-fragmenting activity termed fragmentin-2 was isolated from a rat natural killer cell line and shown to be identical with RNK-P1, the rat equivalent of granzyme B (Shi *et al.,* 1992a). However, fragmentin-2 was only active in inducing DNA fragmentation when perforin was also present. The quantities of perforin required for this synergy with granzyme B were very low, and exposure to the same quantity of perforin alone produced no DNA damage and only barely detectable (usually <5%) specific ^{51}Cr release from the cells. Apoptosis of the target cell proceeded rapidly at 37°C, (>90% maximal DNA fragmentation within 2 h) provided both perforin and granzyme B were added together. Two other fragmentins (-1 and -3) which induced far slower DNA fragmentation in combination with perforin were also identified, and both (corresponding to rat granzyme A and tryptase-2, respectively) were found to possess trypsin-like activity

(i.e., preferential cleavage of substrates at lys or arg residues) (Shi *et al.*, 1992b). The evidence for granzyme involvement in apoptosis induction was strengthened by the observation that the tryptase inhibitor aprotinin could block apoptosis when loaded into the cytoplasm of target cells (Nakajima and Henkart, 1994), and this study provided the first (albeit indirect) evidence that granzymes have to act on *intracellular* substrates within the target cell. Indeed, inactivation of granzyme B by tripeptide (Shi *et al.*, 1992a) or macromolecular (Sun *et al.*, 1996) inhibitors results in inhibition of apoptosis *in vitro*, indicating that proteolytic activity is essential for cytolysis.

The transfection studies of Shiver and Henkart (1991) also provided evidence that the combination of perforin and granzymes could induce target cell DNA fragmentation. In these studies, the rat basophilic leukemia (RBL) cell line acquired cytolytic function following transfection of the gene for rat perforin, but the cells were lytic only when conjugated to target cells via their Fc_e receptors and IgE. Notably, these cells could efficiently lyse erythrocytes but were largely ineffective against nucleated cells (Shiver and Henkart, 1991). However, they acquired the ability to induce apoptosis of nucleated targets (without enhancing their antierythrocyte killing) when they additionally expressed granzyme A and/or granzyme B (Shiver *et al.*, 1992; Nakajima and Henkart, 1994). Transfection of a mouse CTL line with a vector encoding antisense granzyme A mRNA also partially inhibited the lytic activity of CTL (Talento *et al.*, 1992). Finally, the primary *in vitro* activated alloreactive lymphocytes of mice deficient in granzyme B were still able to induce target cell DNA fragmentation, but with far slower kinetics (Heusel *et al.*, 1994). It was notable that in contrast to perforin-deficient mice, these mice were not overly immunocompromised, suggesting that other proteases normally provide a degree of redundancy in the synergy with perforin. The function of perforin, on the other hand, is clearly unique and its absence cannot be compensated, as indicated by the significant T cell immunodeficiency in perforin-deficient mice. The molecular basis for the synergy between perforin and granzyme B is still poorly understood and is the topic of a later section of this review.

3. CL Can Utilize the FasL/Fas Pathway to Elicit Apoptosis

Fas (also known as APO-1 or CD95) is a type I transmembrane protein that was first identified independently by two groups as the molecular target of apoptosis-inducing mouse antibodies of the IgM (anti-Fas) and IgG3 (anti-APO-1) subclasses raised against human tumor cell lines (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). The cloning of Fas showed it to be a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) family of molecules that includes other molecules involved in control of cell proliferation and apoptosis such as CD40, OX40, CD27, 4-1BB, and

CD30 (Nagata and Golstein, 1995). While it was initially suspected that Fas was the receptor for an unidentified cytokine, experiments from the laboratory of Golstein elegantly demonstrated that this molecule could interact with a ligand expressed in an inducible fashion on certain CTL lines (Rouvier *et al.,* 1993). The tenth serial subpassage of a subline of the mouse–rat hybridoma cell line PC60 (PC60-d10s) was incapable of killing via the perforin pathway, but it could efficiently kill target cells transfected to express Fas, though not the parental cell line or control transfectants. A soluble fusion protein incorporating Fas (Fas-Fc), but not one incorporating TNF receptor I, could block this cell death, implying that cell surface FasL played a major role in inducing Fas-mediated apoptosis (Suda and Nagata, 1994). Furthermore, pretreatment of d10S with phorbol 12-myristate 13-acetate (PMA) and ionomycin induced high-level expression of FasL on the effector cells (Yuan and Horvitz, 1992), and repeated sorting of the highest expressing cells resulted in a subline that could lyse Fas-expressing target cells many times more efficiently than the parental d10s cells. It was subsequently shown that soluble Fas fusion proteins could block FasL-mediated killing by a variety of T cell lines (Ju *et al.,* 1994; Stalder *et al.,* 1994). Importantly, this mechanism explained why certain CTL could kill target cells in a calcium-independent manner, (e.g., in the presence of EGTA and $MgCl_2$ which blocks the perforin pathway), or in the absence of perforin, as was observed by Berke and colleagues using PEL as effector cells (see above).

The sum total of cytotoxicity seen in short-term (usually ~4 h) cytotoxicity assays is accounted for by the total effects of perforin- and FasL-mediated mechanisms (Table I). The two mechanisms can operate indepen-

TABLE I
Dual Mechanisms of CL-Induced Apoptosis

	Perforin/granzyme B	FasL/Fas
Principal cells	$CD8^+$ T, NK, $CD4^+$ Th2	$CD8^+$ T, $CD4^+$ Th1
Function	Viral clearance (some), tumor rejection, allograft rejection, NK cytolysis	T cell homeostasis
Calcium dependence	Yes	No
Natural mutation	Not identified	ALPS, gld, lpr
Effect of mutation	Susceptibility to virus, reduced NK, increased tumorigenesis	Autoimmunity, lymphoproliferation
Kinetics of cytolysis	Rapid	Rapid
Caspase dependence	Partial (nuclear effects)	Complete
Mechanism of cytolysis	Apoptosis	Apoptosis

dent of one another, as the FasL mechanism appears to function normally in perforin-deficient CL (accounting for the residual cytotoxicity), while the CTL and NK cells of mice that possess natural mutations of the FasL/Fas mechanism have apparently normal cytolytic granules and express perforin and granzymes in normal amounts (see below). But there is a differential predilection for the two cytolytic pathways by different CL subsets. Granule exocytosis appears to be preferentially used by the $CD8^+$ CTL and NK cells, and this is consistent with the central role of these cells in eliminating virus-infected cells. These so-called "professional killer cells" also use the Fas-based pathway in other circumstances. By contrast, $CD4^+$ T helper cells classically utilize FasL-based cytotoxicity. In particular, Th1 cells readily upregulate FasL expression upon recognition of the target cell and can lyse in a Fas-dependent fashion more readily than Th2 cells (discussed in a later section). As mentioned above, deficiencies of the FasL/Fas mechanism occur in nature, and the phenotype of mice deficient in either receptor (*lpr*) or ligand (*gld*) function is essentially identical within a given strain. In sharp contrast to deficiencies of the granule exocytosis mechanism, these mice do not have an overt T cell immunodeficiency but exhibit abnormalities of T cell homeostatic mechanisms and die of lymphoproliferation and/or autoimmune diseases. Defects of the same mechanism have also recently been defined in humans who present with a remarkably similar syndrome (autoimmune lymphoproliferative syndrome, ALPS), manifesting as severe lymphadenopathy and autoimmune phenomena (Sneller *et al.,* 1992; Illum *et al.,* 1991; Drappa *et al.,* 1996). The implication is that the major role of this mechanism *in vivo* is in regulating T cell development and downmodulating T cell responses following infection. These topics are discussed in greater detail in a later section of this chapter.

4. Granule Exocytosis and FasL/Fas Pathways Trigger an Endogenous Apoptotic Pathway

It was noted in the preceding section that granzyme B is the only mammalian serine protease that cleaves at residues with acidic side chains (Poe *et al.,* 1991). This observation is highly significant in light of recent observations concerning generic mechanisms of apoptosis induction. In short, this type of cleavage specificity appears to have been "set aside" in higher animals for proteases of the serine and cysteine protease families that can both regulate the effector phase of apoptosis and account for much of its characteristic morphology. It is now clear that a family of cysteine protease that share homology with the product of the cell death gene *ced-3* of the nematode *Caenorhabditis elegans* are potent inducers of mammalian apoptosis (Kumar and Lavin, 1996). Three genes, *ced-3, ced-4* and *ced-9* regulate apoptosis in *C. elegans* (Yuan and Horvitz, 1992). *Ced-3* and *ced-4* have a

permissive effect on apoptosis, and inactivation of either gene abolishes apoptosis during nematode development, while *ced-9* is inhibitory for apoptosis and functions by blocking cell death in cells that are required in the adult worm. Ced-9 is similar structurally and functionally to the mammalian Bcl-2 protein (Vaux *et al.,* 1992a), while a mammalian equivalent of the *ced-4* gene product is yet to be identified.

Like granzyme B, the *ced-3* gene encodes a cysteine protease that cleaves target proteins at specific asp residues (Yuan *et al.,* 1993). The cloning of *ced-3* showed it to have strong sequence identity with mammalian interleukin-1β-converting enzyme (ICE), a cysteine protease which was already known to activate IL-1β by cleaving the pro form of the cytokine at asp116 (Kostura *et al.,* 1989; Thornberry *et al.,* 1992). Up to this point, a role for ICE in apoptosis was unsuspected; however, over the the past several years multiple mammalian ICE-like proteases have been identified, cloned, and characterized as playing an integral role in apoptosis induction (Kumar and Lavin, 1996). The burgeoning size of the family has necessitated a new nomenclature, and each enzyme is now known as a "caspase" and given a numerical suffix (Alnemri *et al.,* 1996) (Table II). The mammalian ICE-like proteases have been categorized into three groups based on their sequence similarities, and each falls into the ICE-like, CPP32-like, or Nedd-2-like groups. It is beyond the scope of this chapter to deal with the caspase family in great detail; however, some pertinent features are summarized

TABLE II

ICE-like Proteases, Their Caspase Equivalents, and Cleavage by Granzyme B[a]

Caspase	Original nomenclature	Granzyme B cleavage
1	ICE	No
2	Nedd2/ICH	Yes[b]
3	CPP32	IETD S[c]
4	$ICE_{rel\ II}$/Ch2/Tx	No
5	$ICE_{rel\ III}$/Ty	No
6	Mch2	TEVD A
7	Mch3/ICE-LAP3	IQAD S
8	FLICE/MACH/Mch5	VETD S
9	Mch6/ICE-LAP6	PEPD A
10	Mch4	IEAD A

[a] Table compiled form the following references: Darmon *et al.* (1995), Harvey *et al.* (1996), Srinivasula *et al.* (1996b), Fernandes-Alnemri *et al.* (1996), Quan *et al.* (1995), Gu *et al.* (1996).

[b] The cleavage site in Nedd2 has not been definitively mapped (Harvey *et al.* 1996).

[c] The single-letter amino acid code is used, and the space indicates the cleavage site.

here. Most ICE-like proteases are expressed very widely in different tissues (Kumar and Lavin, 1996). In addition, Nedd-2 is expressed at much higher levels in the mouse embryo (Kumar *et al.,* 1994). All of the proteases are constitutively expressed as single-chained pro proteins that require autocatalysis or cleavage by another protease to achieve the active state. In most cases, the active form consists of two subunits derived from the single precursor by cleavage after specific asp residues. Thus, the typical active caspase is a heterodimer with a heavy chain of approximately 20 kDa noncovalently bound to a light chain of approximately 10 kDa. The crystal structure of ICE (Wilson *et al.,* 1994) has shown it to be a heterotetramer with two heavy chains (residues 120–297 of the pro protein) and two light chains (residues 317–404) (Thornberry *et al.,* 1992). The residues flanking the active site cysteine at position 285 of ICE (typically gln-ala-**cys**-arg/gln-gly) are tightly conserved in all family members, while the residue corresponding to His237, whose imidizole ring is also essential for catalytic activity, is also invariant. Indeed, mutation of either Cys285 or His237 of ICE eliminates both IL-1β production and autoprocessing of ICE (Wilson *et al.,* 1994). All members of the family also carry several asp residues that are the sites of cleavage that generate two subunits analagous to the p20 and p10 chains of ICE (Thornberry *et al.,* 1992; Harvey *et al.,* 1996; Cerretti *et al.,* 1992).

What follows below is a more detailed consideration of the mechanisms underpinning granule exocytosis and FasL-mediated target cell apoptosis. We shall see toward the end of this review that the caspase cascade is required for at least some of the actions of both pathways utilized by CL. Suffice it for now to say that

1. the caspases and granzyme B share the unusual ability to cleave at key specific asp residues of target proteins;
2. through this type of cleavage, an important function of granzyme B is to activate and amplify the caspase cascade;
3. the FasL/Fas mechanism also activates the caspase cascade; and thus,
4. though also maintaining significant functional differences, both the granzyme B/perforin and FasL-mediated apoptotic signaling pathways intersect in the target cell cytoplasm.

B. The Granule Exocytosis (Stimulated Secretion) Mechanism

1. The Nature of Cytolytic Granules

The hypothesis that specialized cytolytic granules deliver quantal amounts of lytic mediators to kill target cells is supported by a vast body of evidence,

though a good deal of it is circumstantial. First, within a few minutes following conjugate formation, the granules become reoriented toward a prospective target (Geiger *et al.,* 1982; Yannelli *et al.,* 1986; Kupfer, 1991). Serine proteases, proteoglycans, and perforin are all then secreted into the intercellular cleft (Schmidt *et al.,* 1985; Takayama *et al.,* 1987; Jenne and Tschopp, 1988), and their binding to the distal membrane can be observed by cinemicrography and electron microscopy (Yannelli *et al.,* 1986). Ring-like tubular lesions measuring up to 18 nm in diameter and apparently identical to those formed by purified perforin are seen to form in the membrane following attack by CTL clones *in vitro* (Dennert and Podack, 1983). Finally, purified granules can elicit the changes of both membranolysis and apoptosis in a dose-dependent manner and with no particular specificity (Tschopp and Nabholz, 1990; Smyth and Trapani, 1995).

The unique appearance of the cytoplasmic granules of activated CTL and NK cells was noted in early electron-microscopic studies, which described unusual dense-cored structures with a well-demarcated peripheral area containing numerous vesicular structures. The granules combine features of two organelles that are usually separate, secretory vesicles and lysosomes (Peters *et al.,* 1991; Burkhardt *et al.,* 1990). The study of Peters *et al.,* (1991) examined lysosomal markers, perforin, and granzymes by immunogold staining and demonstrated that perforin and granzymes were localized within both the dense core and the peripheral regions of virtually all granules. Perforin and granzymes may therefore be liberated from cells as soluble molecules, and additionally in a membrane-encapsulated form. The limiting membrane is rich in lysosomal markers such as CD63, lamp-1, and lamp-2, and these molecules become inserted into the effector cell plasma membrane following membrane fusion during exocytosis. The granules also contained mannose-6-phosphate receptors, which are important for the correct targeting of granzymes during their biosynthesis (see below); however, perforin and other granule constituents such as chondroitin sulfate proteoglycans do not utilize this pathway. Like lysosomes, the granule milieu is acidic (pH approximately 5.5) and the granules are able to communicate with the cell membrane (and can sample the external environment) via endocytosis (Burkhardt *et al.,* 1990). The outer leaflet of the granule vesicles may therefore contain cell surface structures including T cell receptor, MHC, and CD3 (Peters *et al.,* 1989, 1990). The presence of T cell receptors with avidity for target cell MHC/peptide complexes on vesicular membranes has been put forward as one of the many possible explanations for why cytolytic granule proteins (especially perforin) are targeted more or less specifically to the target cell membrane rather than attaching back on the effector cell. The cores of granules are also limited by such a mem-

brane and may therefore also be targeted to the target cell by a similar mechanism.

2. Molecules Present within Cytolytic Granules

a. Perforin

i. Perforin Structure Apart from the NK-like granular metrial cells in the lining of the uterus, perforin is synthesized specifically by CL and is stored within cytolytic granules. The term "perforin" derives from the membrane perforation and resultant osmotic lysis observed in cells exposed either to large amounts of purified perforin or to some *in vitro* derived CTL clones in which very large amounts of perforin have been synthesized and stored. The concept that osmotic cell death can proceed merely from the isolated effects of perforin in any physiological or pathophysiological setting is still the topic of some conjecture, although there is now a broad consensus that the majority of CTL/NK-mediated cell death is through an apoptotic mechanism. Those arguing against the "perforin only" hypothesis of cytotoxicity point out that CTL clones able to kill by osmotic means alone may do so because of their persistent restimulation in the presence of large concentrations of interleukin-2. Therefore, in some instances at least, this type of cell death may represent an *in vitro* artifact. Nevertheless, broad-spectrum caspase inhibitors can be used to demonstrate caspase-independent cell killing in which granzyme-dependent cell membrane damage proceeds in the absence of nucleolysis (Sarin *et al.,* 1997a; Spielman *et al.,* 1997) (discussed further below). Perforin is also known as "cytolysin" (Henkart, 1985), C9-related protein (Zalman *et al.,* 1986a), or PFP (Young *et al.,* 1986a–d; Liu *et al.,* 1986); however, the first of these terms is now by far the most widely used.

Following the demonstration that membranolytic activity resides within cytolytic granules, perforin was isolated, purified to homogeneity, and shown to be capable of forming the same barrel-shaped transmembrane lesions, in a calcium-dependent manner (Podack and Dennert, 1983; Dennert and Podack, 1983; Masson and Tschopp, 1985; Podack *et al.,* 1985; Young and Cohn, 1986). Polyclonal and later monoclonal antisera demonstrated that perforin expression was limited to lymphocytes with cytolytic capability, and the finding that perforin was exocytosed from CTL and could attach to target cell membranes (Podack and Dennert, 1983) argued strongly for the validity of the lytic model of cell death. Because of the obvious similarities between perforin- and complement-induced lesions, their similar migratory properties on SDS–PAGE, and their antigenic cross-reactivity (Tschopp *et al.,* 1986; Young *et al.,* 1986a,d; Zalman *et al.,* 1986a),

it was considered likely that perforin would show strong homology with C9, the final component of the membrane attack complex (MAC) of complement. C9 is the most abundant protein in the MAC (Stanley *et al.,* 1986); however, it can only intercalate the plasma membrane provided earlier proteins of the cascade (C5b, C6, C7, C8a, and C8b) are already in place on the membrane (Tschopp *et al.,* 1986; Young *et al.,* 1986d). Like C9, perforin also migrates more rapidly in SDS–PAGE under nonreducing than under reducing conditions, suggesting a tightly disulfide-bonded structure, which becomes less globular upon disruption of these intrachain links. Perforin is a slightly acidic protein (Pershecini and Young, 1988) and was initially purified by anion exchange chromatography (Podack *et al.,* 1985). Cytolytic granules are easily isolated from cytolytic cell lines such as CTL clones or NK leukemias, or from normal peripheral blood T and NK cells cultured in IL-2. The washed cells are usually disrupted by nitrogen cavitation and the granules are separated from other organelles by density gradient centrifugation, e.g., on substances such as Percoll (Borregaard *et al.,* 1983). More rapid, single-step protocols for perforin purification are now available, for example, using metal affinity chromatography, thus obviating the need for prior density gradient centrifugation to isolate intact granules (Froelich *et al.,* 1996a).

The cloning of the cDNAs encoding mouse (Lowrey *et al.,* 1989; Kwon *et al.,* 1989; Shinkai *et al.,* 1988), human (Lichtenheld *et al.,* 1988; Shinkai *et al.,* 1989), and rat (Ishikawa *et al.,* 1989) perforins predicted the overall similarity to C9 to be surprisingly low. The mouse and human mRNA transcripts migrated as a broad band at approximately 2.7 kb, and the longest open reading frame encoded 555 amino acids for human and 554 amino acids for mouse perforin (open reading frames of 1665 and 1662 bp, respectively). However, both mature proteins are 534 amino acids in length, as the human leader sequence (21 amino acids) is one residue longer than its mouse counterpart. All three perforins have 20 cysteine residues which are completely conserved and are believed to form 10 intrachain disulfide bonds. Human and mouse perforin are ~68% identical at the amino acid level, and mouse and rat perforins are ~86% identical.

The amino-terminal 100 and the carboxy-terminal 150 residues are unique to perforin, although strong evidence has recently emerged that the carboxy-terminal domain is the site of calcium ion binding and initiation of insertion into the lipid bilayer (see below). A stretch of 300 amino acids about the center of the perforin sequence (corresponding approximately to residues 100 to 400) shows a degree of overall identity with the terminal complement components C6–C9 (about 22%). However, two smaller regions within this large central domain have considerably higher similarity to the MAC proteins. The highest degree of homology occurs between residues 190 and 220, which are thought to subserve the function of membrane insertion, as

do the corresponding residues of the complement proteins (Lowrey *et al.,* 1989; Kwon *et al.,* 1989; Liu *et al.,* 1995; Podack, 1989, 1992; Haefliger *et al.,* 1987). The second strongly conserved domain is the region between residues 355 and 388 of perforin, which has similarity to the epidermal growth factor (EGF)-like repeat domains also found in the MAC proteins.

It should be stressed that the functional role of none of the domains of perforin has been clearly demonstrated experimentally, and proposed roles for various regions rest very much on analogy with the complement proteins. It is interesting that this should still be the case so long after the first description of the perforin cDNA sequence (1988); however, a mutagenic analysis of perforin function has not been possible because the systems for expressing recombinant perforin have not been successfully developed (J. A. Trapani, unpublished observations), nor is a crystal or NMR structure of perforin or any of its putative domains yet available. Prokaryotic expression systems have generally produced only insoluble perforin proteins or domains, and expression in eukaryotic cells may lead to toxicity unless the cells are equipped to accurately process and package the resultant polypeptides. Shiver and colleagues utilized the cytoplasmic granules of RBL cells to permit packaging of perforin following transfection, and this bestowed cytotoxicity on this cell line (Shiver and Henkart 1991; Shiver *et al.,* 1992). Liu and colleagues (1996) successfully expressed full-length perforin molecules in insect Sf9 cells using baculovirus vectors; however, the protein lacked potent cytolytic activity, possibly because of faulty processing at the carboxy terminus.

Attachment of perforin to the cell membrane differs from that of C9 in that the presence of other proteins analogous to the MAC proteins in the membrane is not required. In the presence of ~1 m*M* Ca^{2+}, perforin can bind to various lipid molecules directly, provided a phosphorylcholine headgroup is present (Tschopp *et al.,* 1989) and binding is inhibitable by various lipid moieties (Yue *et al.,* 1987). No other receptor molecule has ever been described for perforin, and if such a molecule does exist, one would predict it would have to be both abundant in the cell membrane and ubiquitously expressed. Membrane attachment of perforin occurs equally efficiently at 4°C and at 37°C and is observed with both naturally occurring and synthetic lipid bilayers. Like C9, perforin does not possess a discrete, hydrophobic membrane-spanning domain that is evident from the primary structure. Rather, following attachment to the membrane, a marked conformational change occurs in the molecule, resulting in the exposure of amphipathic alpha-helical regions (residues 190–220). On the basis of molecular modeling, this region is believed to insert into the lipid bilayer by virtue of its contralateral hydrophilic and hydrophobic surfaces. This process is temperature sensitive and occurs optimally at 37°C. Subsequently, like C9, perforin monomers can be added to nascent "barrel-stave" structures, but unlike

heteropolymeric MAC complexes, perforin lesions are homopolymers formed from 12 to 18 coalesced monomers. On the basis of dose-dependence studies, it is likely, however, that only 3 or 4 monomers are sufficient to form a functional channel (Liu *et al.,* 1995). The region that subserves polymerization function is considered likely to be the EGF-like cysteine-rich domain (residues 355–388). Another region of the perforin molecule to have received specific attention is the amino–terminal domain. A synthetic peptide corresponding to the amino-terminal 34 residues of perforin was shown to cause cell lysis of nucleated and nonnucleated cells and liposomes (Ojcius *et al.,* 1991a). Peptides corresponding to residues 189–218 were also able to lyse cells, though less efficiently than the amino-terminal domain. Subsequent studies on the amino-terminal peptide have demonstrated that lytic activity is largely dependent on residues 1–19 and that an 11-residue core (residues 6–16) may form an amphipathic β-sheet, flanked by two short hydrophobic stretches of amino acids. It remains to be seen whether this region is of physiological significance, as baculovirus deletion mutants lacking this region were still able to lyse red cells (Liu *et al.,* 1996).

Some light has recently been shed on the likely function of the carboxy terminus of perforin (Uellner *et al.,* 1997). Until recently, it has not been clear why the lipid-rich membranes of the endoplasmic reticulum and Golgi should not be injured by premature activation of perforin during its biosynthesis. Griffiths and co-workers postulate that perforin is synthesized as an inactive precursor molecule and that final processing to an active form takes place at the carboxy terminus only under the acidic conditions found in lytic granules. Careful pulse-chase experiments showed that the unmodified perforin polypeptide backbone migrates on reducing SDS–PAGE at ~60 kDa (the likely form in the endoplasmic reticulum), and the subsequent addition of complex glycans in the Golgi occurs at two positions (one very close to the carboxy terminus), resulting in a molecule of ~70 kDa. Later again, a smaller "mature" form of ~65 kDa appears in the cytolytic granules. This reduction in size results from a proteolytic cleavage (or trimming) close to the carboxy terminus, and although the exact site of this cleavage is unclear, it appears that a fragment of about 20 amino acids is removed, together with a large carbohydrate moiety attached to it. An acidic granule pH is required for this processing, and neutralization of the lysosomal compartment with ammonium chloride or chloroquine largely ablates cytotoxicity by CTL (Uellner *et al.,* 1997). This mechanism may also explain how perforin can bind calcium ions. The region close to the amino terminus has been shown to have significant homology to the C2-like calcium-binding domains of which the prototype is synaptotagmin, a protein involved in vesicular trafficking and signaling at neuronal synapses, and thought to subserve calcium-dependent lipid binding (Nalefski and Falke, 1996). Following cleavage of the carboxy-terminal region

with its attached carbohydrate, the C2-like domain folds into two, eight-stranded β-sheets, bringing together at a single point a number of aspartate residues which, by virtue of their negative charges, can bind a positively charged calcium ion. Having bound calcium, this region of the molecule is now highly reactive and in the presence of the appropriate lipid moieties can commence the process of attachment and intercalation into the plasma membrane.

ii. Expression of Perforin in Vitro Many early studies examining the expression of perforin utilized relatively insensitive techniques such as assaying for hemolytic activity or immunostaining and Western blotting with relatively low-titered polyclonal antisera, and some of these studies tended to underestimate the numbers of perforin-expressing cells. Even prior to the generation of perforin-deficient animals, it was amply clear that the expression of perforin was very closely associated with the possession or acquisition of cytolytic function. In the mouse and human, all NK cell lines and $CD8^+$ CTL clones express perforin mRNA and protein (Kwon *et al.,* 1989; Ishikawa *et al.,* 1989; Lowrey *et al.,* 1989), as do some $CD4^+$ T helper cells, principally of the Th2 phenotype (Lancki *et al.,* 1991) and γ/δ T cells (Koizumi *et al.,* 1991; Nakata *et al.,* 1990). In unstimulated human peripheral blood lymphocytes, constitutive expression is observed in $CD3^-$ $CD56^+$ NK cells and in two groups of unstimulated $CD8^+$ T cells, the γ/δ TCR^+ cells and α/β TCR^+, $CD11b^+$ subset (Podack *et al.,* 1991; Yagita *et al.,* 1992). "Resting" $CD4^+$ cells are negative for perforin expression (Nakata *et al.,* 1992). The $CD8^+$ $CD11b^+$ cells represent the *in vivo* effector cells, while the $CD8^+$ $CD11b^-$ cells are cytotoxic precursors. Perforin was highly inducible in the $CD11b^-$ population following stimulation through IL-2 or anti-CD3 monoclonal antibodies (mAbs), and 5–20% of $CD4^+$ T cells also acquire perforin expression following similar stimulation (Yagita *et al.,* 1992). Expression of perforin cannot be induced in B cells and monocytes, although it has been observed in monocyte precursors (see below).

In isolated T cells, IL-2 stimulation through the p75 IL-2 receptor is sufficient to induce perforin expression (Smyth *et al.,* 1990a, 1991b). IL-6 (Smyth *et al.,* 1990b; Smyth and Ortaldo, 1991), IL-7 (Smyth *et al.,* 1991a), and IL-12 (Salcedo *et al.,* 1993) are by themselves insufficient to induce expression, but each can synergize with IL-2 to augment it, while TBFβ is inhibitory (Smyth *et al.,* 1991b). The fluid-phase anti-CD3 mAb induces perforin expression in $CD8^+$ T cells in the presence of accessory cells (Liu *et al.,* 1989; Smyth *et al.,* 1990a). Such cells can also be stimulated by an IL-2-independent pathway through calcium ionophores such as ionomycin with or without PMA (Lu *et al.,* 1992). In contrast, PMA/ionomycin fails to induce perforin in the absence of accessory cells (Smyth *et al.,* 1990a). Lectins such as PHA and Con A generally only have minor effects on perforin expression when used in isolation. Negative effects on perforin

expression have been demonstrated for TGFβ, which opposes the actions of IL-2 and IL-6 (Smyth *et al.,* 1991b). There are two reported instances of perforin expression in immature myeloid cells. Perforin can be expressed in $CD34^+$ myeloid precursor cells mobilized in patients treated with GM-CSF and cytotoxic drugs (Berthou *et al.,* 1995). Its expression has also been reported in macrophage precursors that exhibit NK-like lytic activity (Li *et al.,* 1994). Interestingly, the only nonleukocytes which have been shown to express perforin are the granulated metrial gland cells of the uterus of the mouse, which also possess NK-like activity (in humans, these cells are known as endometrial granulocytes), both during the normal menstrual cycle and during pregnancy. The role of these cells is unknown, and perforin-deficient mice demonstrate normal fertility and parity (Kagi *et al.,* 1994a).

iii. Expression of Perforin in Vivo In the days prior to perforin gene knockout mice, pro-perforin protagonists found comfort in the very tight association of perforin expression with pathophysiological situations where CTL-mediated killing was expected, namely, in viral infection, allograft rejection, and autoimmune diseases (see also Hameed *et al.,* 1992). In virus infections such as LCMV, perforin expression occurs at the principal sites of infection (the liver and meninges) and precedes functional cytotoxicity by about 12 h (Muller *et al.,* 1989). Perforin expression by infiltrating CL has been demonstrated in renal (Kataoka *et al.,* 1992) and cardiac (Griffiths *et al.,* 1991) allograft rejection, in rheumatoid arthritis (Griffiths and Mueller, 1991), and viral myocarditis (Young *et al.,* 1990). This latter report is the only one which purports to demonstrate tubular-shaped polyperforin structures on the membranes of damaged cells (in this case on monocyte membranes), and it is not yet clear whether polyperforin pores are absolutely required for inducing apoptotic cell death.

iv. Control of Perforin Gene Expression The perforin gene has been mapped to chromosome 10 in the mouse (Trapani *et al.,* 1990) and to chromosome 17 in the human (Shinkai *et al.,* 1989). Both genes have very simple structures, consisting of only 3 exons and two intervening introns (Trapani *et al.,* 1990; Lichtenheld and Podack, 1989). Exon 1 encodes entirely untranslated sequences, and the entire coding sequence is present on portions of exon 2 and exon 3, which also contains the 3′ untranslated region. The introns are approximately 1.8 and 1.1 kb long in the human, and 1.9 and 2.3 kb in the mouse. The domains of the perforin protein homologous to C9 are not reflected in the intron/exon organization, and indeed the C9 gene organization is very dissimilar to that of perforin, being composed of at least 11 exons spread over approximately 80 kb of DNA (Marazziti *et al.,* 1988).

The levels of perforin expression in a cell are governed largely, if not exclusively, at the level of gene transcription (Garcia-Sanz and Podack, 1993; Lichtenheld and Podack, 1992). A functional analysis of the perforin

gene has demonstrated that *cis*-acting elements may be present for many kilobases upstream of the mRNA start site, suggesting that several, discrete mechanisms may have an effect on perforin gene transcription (Smyth *et al.,* 1994; M. Lichtenheld, personal communication). A relatively small number of dedicated laboratories have tackled the important but difficult area of the transcription factors governing perforin expression. This area is important for at least two reasons. First, the demonstration that perforin is a key molecule in cytolysis has raised the possibility of targeting perforin expression as a means of modulating immune responses, in either a positive or a negative manner. Second, portions of the promoters may be useful for targeting the expression of non-T cell molecules in transgenic animals (Lichtenheld *et al.,* 1995; Smyth *et al.,* 1994). For example, the human granzyme B promoter has been used to direct the expression of human growth hormone to T cells (Hanson *et al.,* 1991). There is a close similarity in the sequences upstream of the human and mouse perforin genes, extending for several kilobases (Trapani and Dupont, 1990). Basal promoter activity has been described for the 300 bp immediately upstream of the transcription initiation site (Koizumi *et al.,* 1993; Smyth *et al.,* 1994; Zhang and Lichtenheld, 1997), and this region contains putative GC boxes and an Ets-binding site related motif. Several further *cis*-acting elements have been identified upstream of this region. The NF-P motif (5′-ACAGGAAG) located at residues −505 to −497 may bind novel Ets-related transcriptional activators. Koizumi *et al.* (1993) have shown that at least two proteins can interact with this motif in mobility shift assays, and while NF-P1 is ubiquitously expressed, the expression of the second, NF-P2, seems to be confined to activated CL. The findings of Koizumi *et al.* have recently been supported and extended by Zhang and Lichtenheld (1997). This group confirmed that a constitutively active transcription factor of the Ets family is expressed and operates in a killer-cell-specific manner through the same region as previously reported. In addition, a number of ubiquitously distributed positive transcriptional elements can operate through regions close to the mRNA start site. In all cell lines examined by Zhang and Lichtenheld, the majority of reporter gene expression resided within 330 bp of the start site. At least three Sp1-like proteins probably bind to two GC boxes in this region (Zhang and Lichtenheld, 1997). Confinement of perforin expression to cytolytic cells is conferred by at least two negatively acting regions which are active in all noncytolytic cells and located about 370 and 650 bp upstream of the initiation site. Thus, even the closer *cis*-acting elements controlling perforin gene expression are a complex mixture of (specific) positive and (broadly expressed) negative influences that ultimately limit expression to CL.

v. Protection from Nonspecific Lysis by Perforin The biosynthesis of toxic granule proteins, especially perforin, poses special problems of self-

protection for CL. We have already seen that perforin biosynthesis results in the initial production of an inactive polypeptide which is activated by proteolytic digestion only at the time of its packaging into lytic granules. This process is regulated largely by the acidic pH optimum of this process. Similarly, the granzymes are synthesized as zymogens which are also activated at the time of inclusion into granules (see below). Much of the calcium within the granule is complexed tightly to calreticulin until degranulation occurs. Polymerized perforin is unable to insert into lipid bilayers, so preactivation by calcium results in a rapid and irreversible loss of activity. Zn ions also inactivate perforin (Podack *et al.,* 1985; Henkart *et al.,* 1984), as do S factor and lipoproteins in serum (Yue *et al.,* 1987), presumably by competition for lipid-binding sites. It is therefore vital that target cell membranes are exposed to perforin in the absence of serum, and the membrane-limited space between the effector and target cells probably provides a tightly regulated molecular environment that is "cleared" of inhibitory influences prior to degranulation.

It is still unclear how the membranes of CL are specifically protected from the lytic effects of perforin. Several hypotheses have been put forward, including rapid repair of polyperforin lesions and the presence of perforin-inhibitory molecules in the plasma membrane (Muller and Tschopp, 1994), present either constitutively or "left over" in the cell surface following fusion of the cell membrane with exocytic vesicles. Killer cells are less susceptible to the effects of both purified perforin and isolated granules, and to the effects of intact CTL (Shinkai *et al.,* 1988; Jiang *et al.,* 1988; Nagler-Anderson *et al.,* 1988; Verret *et al.,* 1987; Blakely *et al.,* 1987). Nevertheless, freshly isolated CTL clones can be potently killed by other CTL (through a fratricidal interaction) when they present a cognate peptide on class I MHC molecules. In one study, electron-microscopic examination of effector/target conjugates suggested that much more perforin attaches to the target than the CL plasma membrane (Ortaldo *et al.,* 1992; J. R. Ortaldo, personal communication). A further possibility is that chondroitin sulfate exocytosed with perforin remains attached to the CL membrane for some time, affording an impermeable barrier back to the CTL (Podack *et al.,* 1991).

Two forms of homologous restriction factor (HRF; one of a family of proteins which protects autologous cells from complement-mediated attack) have also been postulated to block perforin's actions, a 68-kDa form and a 20-kDa form also known as CD59 (Zalman *et al.,* 1986a,b, 1987). The cells of paroxysmal nocturnal hemoglobinuria patients (who congenitally lack glycolipid anchoring of all proteins, including CD59) are exquisitely sensitive to the effects of complement, but show a normal sensitivity to perforin and CTL-mediated cytolysis (Krahenbuhl *et al.,* 1989). Unlike complement, there is no species restriction observed with perforin, that is,

perforin from one species can kill cells of various species equally well (Jiang *et al.,* 1988). Interestingly, however, there is a vast difference in the rate at which individual cells and cell lines can repair perforin lesions (erythrocytes, which cannot repair the lesions at all are very sensitive to perforin). Furthermore, expression of CD59 in target cells affords no protection against perforin or CTL (Podack *et al.,* 1991). It is therefore highly unlikely that HRF is involved in CL-protective mechanisms. Muller and Tschopp (1994), also examined the question of perforin-inhibitory molecules by comparing the ability of radioiodinated perforin to bind to "typical" target cells (K562 human erythroleukemia, P815 mouse mastocytoma cells) and a number of CTL lines. All of these cells bound equivalent amounts of perforin in a reversible manner (following EDTA addition) at 4°C. Warning to 37°C resulted in a similar degree of irreversible attachment to the membrane of both cell types. However, perforin that bound to the resistant cells was cleavable by trypsin into fragments of 55 and 15 kDa, while that attached to susceptible cells was resistant to proteolysis, suggesting that perforin attached to the two cell types was in a conformationally different state. There are several possible interpretations of these data, but Muller and Tschopp postulated the existence of a protein in CTL membranes that could specifically bind to and inhibit perforin. To date, the search for such a molecule has proven fruitless.

vi. Perforin as a Delivery System for Other Apoptotic Mediators Synergy between perforin and other granule components (particularly granzyme B) is required for the apoptotic function of CL and their granules. To explain this synergy, it is intuitively most simple to postulate that perforin-induced membrane fenestrations afford access into the cytoplasm for granzyme B. It is likely that this is an oversimplification of what actually occurs. If perforin could allow access to the cytoplasm in a nondiscriminatory manner, one would predict that (i) other small molecules should diffuse equally well into the target cell and (ii) that other membrane-disruptive agents should be able to synergize with granzyme B to induce apoptosis. Neither prediction appears to be correct. Confocal microscopy of cells exposed to perforin and fluorescein-conjugated purified granzyme B has shown that while perforin does accelerate the uptake of granzyme B into the cell, much of the uptake of granzyme B occurs independent of perforin (Trapani *et al.,* 1996, 1997; Shi *et al.,* 1997; J. A. Trapani and D. A. Jans, unpublished observations; R. C. Bleackley, personal communication). Furthermore, inert and freely diffusible molecules as small as 13 kDa are not appreciably taken up in the presence of the same quantities of perforin that accelerate the uptake of granzyme B (J. A. Trapani and D. A. Jans, unpublished observations). This argues against both the free diffusion of molecules though polyperforin pores and equally the random uptake of extracellular molecules through endocytic repair of perforin-induced le-

sions. We have been unable to find membrane-disruptive agents that can substitute for perforin in synergizing with granzyme B. Complement lesions do not suffice, nor do lesions formed by a variety of detergents (Shi *et al.*, 1997). Suffice it to say that the collaboration between perforin and granzyme B is highly specific. The issue of perforin/granzyme synergy is taken up once again in a later section of this chapter.

b. Granzymes A family of lymphocyte-specific serine proteases termed "granule enzymes" or "granzymes" (Tschopp and Jongeneel, 1988) comprises about 90% of the protein within cytolytic granules (Henkart *et al.*, 1987; Ojcius *et al.*, 1991b). The granzymes are related to the chymotrypsin family of serine proteases and demonstrate structural similarities and genetic linkage to other leukocyte serine proteases, especially those expressed in mast cells and monocytes. The key feature of serine proteases is their dependence for catalytic activity on an active site serine residue, one of a triad of catalytic residues corresponding to His^{57}, Asp^{102}, and Ser^{195} in chymotrypsin (Smyth *et al.*, 1996a,b). Other requirements include an oxyanion hole to stabilize transition states of the enzyme–substrate complex and a substrate-binding pocket, the configuration of which determines the specificity the individual serine protease (Kraut, 1977).

i. Cloning of Granzyme Genes The observation that protease inhibitors could block cytotoxicity (Chang and Eisen, 1980; see also Hudig *et al.*, 1991; Woodard *et al.*, 1994) was followed by a search for proteases that might be involved in the killing process. Pasternack and Eisen first reported specific serine protease activity emanating from CTL (Pasternack *et al.*, 1986). The first reports of CL-specific serine protease genes in the mouse were from the laboratories of Bleackley (Lobe *et al.*, 1986a,b), Golstein (Brunet *et al.*, 1986), and Weissman (Gershenfeld and Weissman, 1986). These groups utilized mRNA competition or subtraction approaches to isolate transcripts that were specifically expressed in CL. The Bleackley group isolated two new cDNAs, CCPI and CCPII, which encoded related serine proteases expressed in cytolytic but not in helper mouse T cell clones. CCPI was later found to correspond to granzyme B, and CCPII to granzyme C, using the nomenclature devised by the Tschopp group, and based on biochemical characterization of granule proteases (Masson and Tschopp, 1987; Tschopp and Jongeneel, 1988). Gerschenfeld and Weissman also isolated a granzyme A sequence and named it "Hannukah factor," based on its similarity to Christmas factor (blood clotting factor IX), a non-CTL serine protease (Gershenfeld *et al.*, 1988). The clone isolated by Brunet and colleagues (CTLA-1) also encoded mouse granzyme B.

Over the next several years, a multiplicity of publications appeared that reported the cloning of cDNA sequences for mouse, human, and rat gran-

zymes (Table III). A variety of cloning strategies were used, including subtractive hybridization, protein purification and partial amino acid sequencing, the use of heterologous nucleotide probes under low stringency, and, most recently, polymerase chain reaction amplification using primers corresponding to conserved patches of sequence. These predicted sequences were matched with the proteins and proteolytic activities identified in careful biochemical characterization (Masson and Tschopp, 1987; Sayers *et al.*, 1992, 1994). All in all, a total of eight granzymes (A–G and M) have been identified in the mouse; however, only five are identified in humans (A, B, H, M, and protease-3). The rat shares granzymes A, B, C, F, M, and tryptase-2 in common with humans and mice but in addition expresses granzyme J (Ewoldt *et al.*, 1997a) and two other proteases of uncertain specificity designated RNK-P4 and RNK-P7 (Ewoldt *et al.*, 1997b). No human equivalents of mouse granzymes C–G have been identified, while granzyme H appears to be specifically expressed by human cells (Smyth and Trapani, 1995; Trapani and Smyth, 1993).

ii. Granzyme Biosynthesis and Processing Like many serine proteases in other systems, granzymes are produced as inactive precursor molecules (zymogens) and are fully processed only at the time of packaging into the lytic granules. The nascent granzyme polypeptide is a pre-pro protein that is equipped with a typical leader sequences to enable transport through the endoplasmic reticulum and Golgi. Leader cleavage generally leaves two amino acids attached to the mature amino terminus, based both on predictive models (von Heijne, et al. 1986), which show favorable cleavage consensus sequences at the -3/-2 positions and experimental evidence (Caputo *et al.*, 1993; Smyth *et al.*, 1995b). In COS-7 cells, which normally cannot process granzyme protein, transient expression of granzyme cDNA constructs lacking the final two amino acids of the leader sequence resulted in active granzyme B, while a matched construct possessing an unmutated leader resulted in equal amounts of inactive protein (Smyth *et al.*, 1995b). It is likely that the final activation pro dipeptide is normally clipped from the remainder of the polypeptide by dipeptidyl peptidase I (DPP1; also known as cathepsin C), an enzyme expressed by myeloid cells and lymphocytes that also express granzyme-like serine proteases (McGuire *et al.*, 1993). Cathepsin C/DPP1 is a lysosomal enzyme which is colocalized within the lysosome-like secretory vesicles of CL, enabling granzyme activation immediately prior to packaging (Brown *et al.*, 1993). In view of the potent effects of some granzymes on activating apoptotic pathways, it is clearly desirable to limit access of active granzymes to the cytoplasm. The pH optimum of granzymes is ~7.5, and optimal activity is not reached until release from the secretory granules. By contrast, the pH optimum for DPP1 is 5.5 (McGuire *et al.*, 1993; Smyth *et al.*, 1995b), the approximate intragranular pH. If DPP1 is the only enzyme capable of this final processing step, this

TABLE III

Human and Rodent Granzymes[a]

Protease	Species[b]	Other names	Enzyme activity	Predicted cleavage	CHO[c]	diS[d]	−SH[e]
Gr A	MHR	HF, MTSP-1, SE-1, CTLA-3	Tryptase	R/K[f]	1	4	1
Gr B	MHR	CCP-1, CTLA-1, RNK-P1, HLP, granzyme 2, HSE 26.1, CSPB, fragmentin-2	Aspase	D/E	1/2[g]	3	0/1[h]
Gr C	MR	CCP-2, RNK-P4	Unknown	N/S	0	3	0
Gr D	M	CCP-5	Unknown	F/L	5	3	0
Gr E	M	CCP-3, MCSP2	Unknown	F/L	4	3	0
Gr F	MR	CCP-4, MCSP3	Unknown	F/L	3	3	1
Gr G	M	MCSP1	Unknown	F/L	3	3	0
Gr H	H	CCP-X, CSP-C	Unknown				
Gr J	R		Unknown				
Gr M	MHR	RNK-Met-1, Met-ase	Met-ase	M/L	2	4	0
Unassigned	R	Fragmentin-3[i], tryptase-2[i]	Tryptase	R/K			
Unassigned	H	Granzyme 3[j]	Tryptase	R/K			
Unassigned	R	RNKP-7	Unknown	F/L			

[a] Compiled from Brunet *et al.* (1986), Bleackley *et al.* (1988), Gershenfeld *et al.* (1988), Gershenfeld and Weissman (1986), Haddad *et al.* (1991), Jenne *et al.* (1988a,b, 1989), Kelly *et al.* (1996), Kwon *et al.* (1988), Lobe *et al.* (1986a,b, 1988), Sayers *et al.* (1994), Trapani *et al.* (1988), Schmid and Weissmann (1987), Smyth *et al.* (1992, 1993), Klein *et al.* (1989), Zunino *et al.* (1988, 1990). Modified from Hudig *et al.* (1993) and Smyth and Trapani (1995) with additions.

[b] M, mouse; H, human; R, rat.

[c] Carbohydrate addition sites (putative, N-linked).

[d] Intrachain disulfide bonds.

[e] Predicted unpaired cys residues.

[f] Single-letter amino acid code.

[g] Mouse and human have one, rat two.

[h] Mouse and human have one, rat none.

[i] Fragmentin-3 and tryptase-2 are almost certainly synonymous, and the homolog of granzyme 3.

enzyme is clearly at a pivotal position in terms of regulating granzyme function. Expression of granzymes with an unmutated leader sequence in cells lacking DPP1 results in the production of inactive granzyme protein. Alternatively, granzymes can be engineered for expression in such cell types, if the activation dipeptide is deleted by recombinant DNA techniques (Caputo *et al.,* 1993; Smyth *et al.,* 1995b). It is not clear whether the pro forms of all granzymes involve a dipeptide, as the sequence at the -3/-2 positions of some granzymes is not favorable according to von Heijne's criteria, while a better consensus is present at -7/-6. Indeed, deletion of the final six amino acids from the encoded leader sequence resulted in superior granzyme M activity in COS-7 cells, compared with deletion of just the dipeptide (Smyth *et al.,* 1995b). Interestingly, the position of intron 1 in granzyme genes seems to correlate with the most favorable cleavage site (Smyth *et al.,* 1996c). If processing of an activation pro hexapeptide is physiological, the enzymes responsible for it are as yet unidentified.

Targeting of granzymes to the lytic granules occurs in a manner analagous to that of some other lysosomal enzymes. Granzymes A and B, for instance, are glycosylated with mannose-rich carbohydrates containing mannose-6-phosphate moieties which are important for accurate packaging through the mannose-6-phosphate receptor pathway (Griffiths and Isaaz, 1993). However, a mannose-6-phosphate-independent pathway also exists and accounts for a minority of the granzyme protein ultimately packaged. Low pH conditions (e.g., the pH of the trans-Golgi stacks is about 5) are conducive for aggregation of perforin, granzymes, and chondroitin sulfate proteoglycans into multimeric complexes that may then be wrapped in Golgi membranes and fused with existing granules (Masson *et al.,* 1990). Furthermore, the glycosylation of the different granzymes is very heterogeneous. Granzymes undergo N-linked, rather than O-linked, glycosylation. Mouse granzyme C appears to have no glycosylation, while other granzymes such as human granzyme B undergo varying degrees of glycosylation, with a number of species identified between 26 and 32 kDa (Trapani *et al.,* 1993). At the other end of the spectrum, granzyme D has half of its molecular weight made up of carbohydrate addition at up to five asparagine residues.

iii. Granzyme Structure and Proteolytic Specificity Granzymes display many features in common with in other chymotrypsin-like enzymes but also have several defining characteristics of their own (Smyth *et al.,* 1996a):

1. They have highly conserved residues at positions 1–4 (typically ile-ile-gly-gly) and 9–16;
2. Their propeptide sequence is generally gly-glu or glu-glu (Murphy *et al.,* 1988; Caputo *et al.,* 1993);
3. They generally have three conserved disulfide bridges. Exceptions to this rule are granzymes A, 3, and M, which have four intrachain linkages.

Granzyme A is also the only member of the family to exist normally as a dimer, due to an additional unpaired cys residue that takes part in interchain disulfide bonding (Hudig *et al.,* 1993). Mouse and human granzyme B also have an unpaired cys; however, naturally occurring dimers have not been described.

4. Finally, the granzymes have unusual substrate preferences. Serine proteases such as trypsin, chymotrypsin, and elastase generally cleave at basic (lys, arg) residues (hence, "tryptase" activity) or at bulky nonpolar (phe, trp) residues ("chymase" activity), respectively. Not surprisingly, these degradative enzymes have a very broad range of physiological substrates, i.e. the surrounding amino acid context of the residue at the P1 position appears to be far less critical than that for granzymes, in which as many as four neighboring residues may influence recognition (Odake *et al.,* 1991). For example, granzyme B was found to cleave the substrates Boc-ala-ala-asp-Sbzl, Boc-ala-phe-asp-Sbzl and Boc-phe-ala-asp-Sbzl with moderate efficiency; however, the substrate Z-asp-Sbzl was not cleaved (Odake *et al.,* 1991). This exactness of proteolytic cleavage is consistent with a role for granzymes in processing rather than degrading target proteins. Although early attempts to purify granzymes to homogeneity often resulted in loss of activity (Hudig *et al.,* 1993), clear substrate specificites have been identified for granzymes A and 3 (tryptases, often measured as cleavage of BLT) and for granzymes B (an "Asp-ase," cleaving at asp and possibly glu) and M (also known as "Met-ase"). Granzyme A cleaves at arg preferentially to lys (Odake *et al.,* 1991). Granzyme B is the only known mammalian serine protease with preference for acidic side chains (Poe *et al.,* 1991), and the significance of this observation for its role in apoptosis has been mentioned above and will be topic of further discussion below. By contrast, granzyme M is expressed only in NK cells and cleaves preferentially at long, unbranched hydrophobic residues such as met, leu, and nor-leu residues (Sayers *et al.,* 1992; Smyth *et al.,* 1992, 1996a). Granzymes C–G are predicted to be chymases and preferentially cleave synthetic substrates with phe, leu, or asn in the P1 position (Odake *et al.,* 1991).

Residues corresponding to 189, 214–216, and 226 in chymotrypsin are important for forming the substrate-binding pocket and therefore have a large effect on determining the type of amino acid side chain that can be cleaved (Kraut, 1977). Although the crystal structure of none of the granzymes has yet been reported, molecular modeling based on the known structure of chymotrypsin, elastase (Bode *et al.,* 1989), and rat mast cell protease II (Remington *et al.,* 1988) has permitted a detailed analysis of the substrate-binding pockets of granzymes B and M, and these predictions have been validated by mutational analysis (Smyth *et al.,* 1996a; Caputo *et al.,* 1994). Recombinant human granzyme M expressed in COS-7 cells

cleaved thiobenzylester substrates with met in the P1 position (Smyth *et al.,* 1996a). When two residues flanking the substrate pocket, lys179 and ser201, were mutated, respectively, to met and gly (the corresponding residues of chymotrypsin), granzyme M lost met-ase activity but acquired chymase-like activity for substrates with phe at P1. Based on molecular modeling, it is postulated that hydrogen bonding between lys179 and ser201 effectively restricts the substrate cleft of granzyme M to accept only long, narrow side chains, while the residues lining the catalytic pocket are analogous to those of chymotrypsin and can therefore interact with either type of substrate.

A similar analysis has been carried out for granzymes A and B, based on similarity with the trypsin and rat mast cell protease II structure, respectively (Murphy *et al.,* 1988). Granzyme A, like bovine trypsin, has an asp residue at position 189 in the substrate pocket, consistent with a requirement for a basic residue in the P1 position of the substrate. The residue at position 99 is also in the substrate pocket and may interact with the P2 residue. Key residues in determining the substrate specificity of granzyme B include ala177, ser198, tyr199, gly200, and arg208. The side chain of arg208 is probably oriented toward the active site, consistent with a predilection for acidic residues, particularly asp, which has a short side chain. This model is consistent with the results of Poe *et al.,* (1991), who found only weak cleavage of synthetic substrates with glu at the P1 position. Granzyme B can also weakly cleave substrates with asn or ser at the P1 position, probably through hydrogen bonding of these residues with arg208. Consistent with these predictions, mutation of arg208 to gly208 abrogated asp-ase activity and resulting in the acquisition of chymase activity (Caputo *et al.,* 1994). In addition, position 99 in granzyme B is arg, suggesting that an acidic residue may ideally be at P2 in the substrate.

iv. Some Additional Physical Features of Granzymes A and B As stated above, mouse and human granzyme A are disulfide-linked homodimers with two active sites (Gurwitz *et al.,* 1989; Masson *et al.,* 1990), due to the use of a supernumary cys residue for interchain bonding. Under reducing SDS–PAGE analysis, the apparent molecular mass shifts from ~60 to ~30 kDa (Simon *et al.,* 1986; Fruth *et al.,* 1987). Active mouse granzyme A has 232 amino acids (Gershenfeld and Weissman, 1986) and human granzyme A has 234 amino acids (Gershenfeld *et al.,* 1988). Both have four, rather than three intrachain disulfide bonds. Like other granzyme genes, the mouse granzyme A transcript is made up from five exons, the first of which encodes untranslated sequences, while exons 2, 3, and 5 encode individual amino acids of the catalytic triad. Unlike the other granzymes, however, there are two granzyme A mRNAs that arise from the alternative splicing of two different exons 1 (Hershberger *et al.,* 1992). The functional

significance of the two mRNAs is unclear. Because of the close similarity in size, only a single species is usually apparent on Northern blots.

A variety of synthetic compounds encompassing peptide thiobenzyl ester (SBzl), 7-amino-4-methylcoumarin (AMC; Odake *et al.,* 1991), and paranitroanilide (pNA; Simon *et al.,* 1986) derivatives have been tested to determine optimal substrates and cleavage conditions for granzymes, including granzyme A. The optimal pNA substrates found is D-Pro-phe-arg-pNA for mouse granzyme A and Tosyl-gly-pro-arg-pNA for human granzyme A. The pH optimum for cleavage of pro-phe-arg substrates is 7.5 to 8.5 (Simon and Kramer, 1994). Both mouse and human granzyme A are susceptible to inhibition by serine protease inhibitors such as diisopropylfluorophosphate, phenylmethylsulfonylfluoride, bezamidine, aprotinin, leupeptin, and soybean trypsin inhibitor (Table IV). Granzyme A is not dependent on Ca^{2+} or Mg^{2+}; indeed, high concentrations of Ca^{2+}, Zn^{2+}, or reducing agents

TABLE IV
Granzyme Inhibitors

Inhibitor	Granzyme A[a]	Granzyme B[b,c]
Boc-ala-ala-asp-CH_2Cl	−	+
D-pro-phe-arg-CH_2Cl	+	−
N-α-Cbz-L-Phe-CH_2Cl	−	−
Z-val-ala-asp-fmk	−	−
Asp-glu-val-asp-fmk	−	−
DFP	+	+
PMSF	+	−
Benzamidine	+	−
Soybean trypsin inhibitor	+	+
Aprotinin	+	−
Leupeptin	+	−
α_2-Macroglobulin	+	+
Antithrombin III	+	−
α_1-Antiprotease inhibitor	+	+
Phosphoramidon	NR[d]	+
Chymostatin	NR	±
Antipain	NR	±
Elastatinal	NR	−
Pepstatin	NR	−

[a] Compiled from Simon and Kramer (1994).

[b] Compiled from Peitsch and Tschopp (1994) and Poe *et al.* (1991).

[c] For granzyme B: +, >50% inhibition of activity at the reported concentrations; ±, 20–50% inhibition; −, <20%.

[d] NR, not reported.

such as dithiothreitol can inhibit activity (Simon *et al.,* 1986). In addition, granzyme A activity can be blocked by a number of physiological protease inhibitors, such as α_2-macroglobulin, antithrombin III (Masson and Tschopp, 1988), and C1 esterase inhibitor, which may play a role in protecting local tissues from bystander damage following degranulation (Simon and Kramer, 1994).

Many of the compounds that inhibit granzyme A have only marginal effects on granzyme B (Table IV). For example, benzamidine, leupeptin, pepstatin, and antithrombin III have no inhibitory effect (Poe *et al.,* 1991), while soybean trypsin inhibitor, aprotinin and α_2-macroglobulin are only moderately inhibitory. In one extensive study, the best inhibitor identified was human α_1-protease inhibitor, which produced 85% inhibition when used at 10 μg/ml (Poe *et al.,* 1991). The Boc-ala-ala-asp-SBzl esterase activity of granzyme B is seen in the pH range 6.0 to 8.0 (maximal at 7.5) and is enhanced by the presence of 50 m*M* $CaCl_2$ (Peitsch and Tschopp, 1994).

v. Granzyme Subfamilies Based on Genetic Linkage and Gene Organization Gene mapping studies have previously shown that human and mouse granzyme subfamilies are distributed to three corresponding loci, and it is noteworthy from the evolutionary viewpoint that each subfamily seems to constitute essentially a single broad type of substrate specificity (Table V). In the human, granzymes A and -3 map to chromosome 5q11-q12 (*HFSP* and *TRYP2,* respectively; Baker *et al.,* 1994b), granzymes B and H map within a cluster of leukocyte serine protease genes at 14q11 that also includes the gene for the myeloid cell protease cathepsin G (Salvesen *et al.,* 1987; Harper *et al.,* 1988; Hanson *et al.,* 1990), while granzyme M is located within a further cluster of genes also encoding azurocidin (*AZU*), neutrophil elastase (*NE*), and proteinase-3 (*PR3*) on chromosome 19p13.3 (Baker *et al.,* 1994b; Pilat *et al.,* 1994). In the mouse, the corresponding chromosomal assignments are chromosomes 13D (granzyme A and other tryptases) (Mattei *et al.,* 1987), 14D (granzymes B–F) (Jenne *et al.,* 1988a, 1989), and 10C (granzyme M, *AZU,* and *NE*) (Thia *et al.,* 1995). Broadly speaking, these subfamilies represent trypsin-like, chymotrypsin-like, and elastase-like specificities, respectively (Smyth *et al.,* 1996c). The individual members of these clusters of genes have their own organizational peculiarities; for example, the Met-ase, AZU, and PR3 all have intron 1 mapping between the residues -7 and -6 of the leader sequence, indicating a close evolutionary relationship. The genes encoding granzymes B and H and cathepsin G are particularly closely linked, mapping to within 50 kb of each other, and are also close to the loci for the T cell receptor α and δ genes (Hanson *et al.,* 1990). The granzyme H-encoding gene is approximately midway between the other two genes and appears to have arisen from duplication and gene conversion events to produce a "hybrid" gene consisting of the first two exons of an ancestral gene related to mouse granzymes C–G and the

TABLE V
Mouse and Human Granzymes by Grouped Chromosomal Loci[a]

Chromosomal localization	Species	Granzyme(s)	M_r[b]	Lymphocyte expression
The "tryptase" locus				
5q11-q12	Human	A	60	CTL, NK
		Tryptase-2	30	CTL, NK
13D	Mouse	A	60–70	CTL, NK, γ/δ[c], thymus
The "chymase" locus				
14q11-q12	Human	B	29–32	CTL, NK, γ/δ, thymus
		H		CTL, NK
14D	Mouse	B	29–33	CTL, NK
		C	27	CTL
		D[d]	35–50	CTL
		E	35–45	CTL
		F	35–40	CTL
		G[e]	30	CTL
The "metase" locus				
19p13.3	Human	M	30	NK
10q21.2	Mouse	M	30	NK

[a] Compiled from Baker *et al.* (1994a,b), Crosby *et al.* (1990), Fink *et al.* (1993), Jenne *et al.* (1991), Klein *et al.* (1990), Mattei *et al.* (1987), Smyth *et al.* (1993, 1995c), Zimmer *et al.* (1992), and Thia *et al.* (1995).

[b] $M_r \times 1000$.

[c] Gamma-delta TCR^+ T cells.

[d] Granzyme D has not been definitively mapped but is highly homologous with granzymes E and F.

[e] Granzyme G has not been definitively mapped but is highly homologous with granzyme B.

remainder of the granzyme B gene, followed in turn by the accumulation of random mutations (Haddad *et al.*, 1991).

vi. Expression of Granzymes The expression of granzymes has been shown in a variety of studies to be restricted to activated, mature T lymphocytes, thymocytes, γ/δ T cells, and NK cells (Masson *et al.*, 1985; Garcia-Sanz *et al.*, 1987; Ebnet *et al.*, 1991). NK cells and γ/δ T cells constitutively express and store granzymes; however, granzyme mRNA and proteins must be induced in T lymphocytes following exposure to antigen or following other types of stimulation. Granzymes are expressed by most $CD8^+$ and a smaller proportion of $CD4^+$ T cells sensitized *in vitro* by antigen or lectin (Liu *et al.*, 1989).

One extensive study in the mouse (Garcia-Sanz *et al.*, 1990) used the sensitive technique of RNase protection to detect specific mRNA transcripts

for mouse granzymes A to G in lymphoid and nonlymphoid tissues. Unstimulated peripheral T cells did not contain detectable granzyme transcripts; however, all granzyme genes were induced with Con A/IL-2, which led to proliferation of >95% $CD8^+$ T cells. However, administration of PMA/IL-2 to lymph node T cells resulted in the expression of only granzymes A, B, and C. Treatment with PMA/ionomycin resulted in mRNA for granzymes B, C, E, and G. All of the granzyme mRNAs were expressed in alloantigen-stimulated $CD8^+$ and $CD4^+$ T cells. Only granzymes A and B were expressed in PEL, and granzyme A transcripts alone were detectable in whole thymic mRNA. Cultured $CD4^-8^-$ thymocytes (both α/β^+ and γ/δ^+) expressed all granzyme mRNAs upon stimulation, and this finding was in keeping with a similar study (Held *et al.*, 1990a) that suggested granzyme A expression might be important in T cell development. Non-specific IL-2-stimulated T cells also express granzymes (Manyak *et al.*, 1989; Trapani *et al.*, 1988). Nonlymphoid tissues other than lung and intestine did not express any granzyme genes, and this form of expression was put down to contaminating lymphocytes. The only other exception to this rule is that the uterine granular metrial cells express granzyme A in addition to perforin (Zheng *et al.*, 1991). The study performed by Garcia-Sanz *et al.* predated the discovery of granzyme M, and it was subsequently shown in the rat that expression of this granzyme is restricted to NK cells, particularly to the large granular lymphocyte, rather than the small, dense NK cells (Smyth *et al.*, 1992). *In vivo,* granzyme expression (particularly granzyme A) has been described in the T cells infiltrating allografts (Muller *et al.*, 1989) and in pancreatic islets during the onset of autoimmune diabetes mellitus (Held *et al.*, 1990b; Griffiths and Mueller, 1991). Granzyme A protein has also been detected in the granules of T cells during viral infection (Kramer *et al.*, 1989) and in skin lesions during *Leishmania* infection (Moll *et al.*, 1991). In a further study that examined heterotopic cardiac transplantation, the progress of rejection correlated closely with the numbers of granzyme A^+ T cells infiltrating the grafts, and when indefinite graft survival was induced with either cyclosporine or anti-CD4 mAbs, virtually no granzyme A^+ cells were detectable (Mueller *et al.*, 1993; Chen *et al.*, 1993). Similar studies concluded that perforin and granzyme mRNA expression could be used as sensitive markers of graft rejection (Lipman *et al.*, 1992).

In human disease states, the specific expression of granzyme mRNA or protein was first shown in a number of skin diseases (Wood *et al.*, 1988), in renal allograft rejection (Clement *et al.*, 1990), and in HIV-infected lymph node cells (Devergne *et al.*, 1991). There have been several studies of granzyme expression in rheumatoid arthritis (Griffiths *et al.*, 1992; Young *et al.*, 1992; Tak *et al.*, 1994), and in most instances, expression of granzymes and perforin in synovial tissue has been in NK cells, especially early in the disease (Tak *et al.*, 1994). Hameed and colleagues (1991) produced

polyclonal antibodies detecting granzyme B and showed that rejecting cardiac tissues are infiltrated with granzyme B-expressing cells (Clement *et al.,* 1991). Granzyme A expression has also been detected in *in vivo* activated T lymphocytes in rejecting heart allografts (Griffiths and Mueller, 1991; Young *et al.,* 1990). Overall, it is fair to say that granzyme expression is a useful marker of human allograft rejection. Indeed, in a study that examined the expression of various cytokine genes and granzyme genes, the correlation between rejection and granzyme expression was far stronger than that with cytokine or IL-2 receptor expression (Lipman *et al.,* 1992).

vii. Functions of Granzymes Other Than in Apoptosis A large part of this review has dealt with the likely role of granzymes, particularly granzyme B and to some extent granzyme A, in eliciting target cell DNA fragmentation and apoptosis. A number of additional putative functions have also been assigned to granzymes, especially granzyme A:

1. It has been suggested that granzyme A may be involved in regulating B cell proliferation, and purified granzyme A, like the tryptases thrombin and trypsin, can be mitogenic for B cells in the absence of antigenic stimulation (Simon *et al.,* 1986).
2. Granzyme A can cleave several extracellular matrix proteins, and it may therefore facilitate T and NK cell migration through the subendothelial matrix (Simon *et al.,* 1987b; Simon and Kramer, 1994). These putative substrates include proteoglycans and type IV collagens (Simon *et al.,* 1991), laminin (Young *et al.,* 1986b), and fibronectin. Exocytosis of granzyme A can be facilitated by engagement of cell surface integrin molecules with extracellular matrix proteins (Takahashi *et al.,* 1991), an observation consistent with this hypothesis. In addition, granzyme A can activate the pro-urokinase-like plasminogen activator, which should enable it to recruit the extracellular matrix degradative capacity of plasmin (Brunner *et al.,* 1990; Simon and Kramer, 1994).
3. The expression of granzymes in immature thymocytes has been taken to indicate the possible involvement of granzymes in thymic development (see above).
4. Granzymes can induce cytokine secretion and directly activate certain cytokines, thereby potentially amplifying a local inflammatory reaction. Part of this effect is mediated through the thrombin receptor which can be cleaved on a variety of cells at the sequence leu-asp-pro-arg*-ser, with the asterisk indicating the point of trypsin-like cleavage. This can result in diverse effects including the release of IL-6 and IL-8 from monocytoid cells, and the retraction of neurites in glial cells, particularly oligodendrocytes (Suidan *et al.,* 1994). In addition, granzyme A, but not granzyme B, has recently been shown to activate pro-IL-1β, i.e., granzyme A is an ICE (Irmler *et al.,* 1995), although the physiological relevance is unclear.

5. A possible direct role for granzyme A in controlling viral infection has been proposed on the basis that granzyme A can cleave proteins that are essential for viral replication, including reverse transcriptase and the envelope protein gp70 from the Moloney murine leukemia virus (Simon *et al.,* 1987a; Simon and Kramer, 1994). Thus, exocytosed granzyme A might directly alter the infectivity of virus particles budding from infected cells. In a landmark finding, mice with a targeted disruption of the granzyme A gene were found to be profoundly susceptible to infection with the cytopathic orthopox virus ectromelia (Mullbacher *et al.,* 1996), despite being capable of a normal response to the noncytopathic virus LCMV, the intracellular bacterial pathogen *Listeria monocytogenes,* and to eradicate syngeneic tumors with kinetics similar to those of wild-type littermates (Ebnet *et al.,* 1995). Interestingly, cytolytic activity toward ectromelia-infected cells was normal in these mice, as were the kinetics of CTL induction (Mullbacher *et al.,* 1996). The reason for increased susceptibility to ectromelia remains unclear. In a more recent and detailed study, Simon *et al.,* (1997) compared the responses of granzyme A-deficient mice to LCMV, ectromelia, and influenza infection. The findings for LCMV and ectromelia supported those described above, and granzyme A was concluded to be dispensible for recovery from LCMV and beneficial in fighting ectromelia. Fascinatingly, intranasal application of a moderate dose of influenza virions resulted in a protective effect seen in the granzyme A-*deficient* mice. By Day 3, the NK infiltrate in the lungs of both types of animal was similar; however, by Day 6 the knockout mice had an increased viral load, and fewer circulating influenza-specific CTL. Despite these apparent handicaps, the mice survived better because they lacked the severe inflammatory response seen in the lungs of wild-type mice. It is likely that this effect was mediated by granzyme A inducing the release and activation of IL-1 and possibly IL-6 and IL-8, resulting in reduced alveolar gas transfer (see point 4, above). Granzyme A can therefore be considered detrimental to the mice during the recovery phase of infection.

6. Granzyme B has been shown to inhibit the proliferation of a tumor cell line *in vitro* by preventing its adhesion to extracellular matrix proteins via cleavage at the arg-gly-asp binding motif (Sayers *et al.,* 1992). This type of cleavage also has the potential to enable disengagement of the effector cell from the target.

7. Granzyme A, but not granzyme B, has been shown to be capable of binding and cleaving the nuclear protein nucleolin; however, the significance of this observation remains unclear (Pasternack *et al.,* 1991). The nuclear targeting of granzymes in permeabilized hepatoma cells is not affected by anti-nucleolin antibodies, suggesting that nuclear transport of the granzymes is not dependent on nucleolin's movement across the nuclear membrane (see below; J. A. Trapani and D. A. Jans, unpublished data).

8. Hudig and colleagues (1993) have argued that a chymase protease (presumably a granzyme) is important for the activation of perforin and for the acquisition of its lytic activity. This group argues that most preparations of perforin are contaminated with chymases and that these preparations can be rendered nonlytic by protease inhibitors such as sulfonylfluorides and chymase-directed phosphonates (Ewoldt *et al.*, 1992). This hypothesis has not received widespread support, but it needs to be reconsidered in view of the recent demonstration that perforin requires cleavage close to its C terminus to achieve activity at the appropriate moment in its biosynthesis (Uellner *et al.*, 1997). The protease responsible for this activation has not yet been elucidated.

C. Other Molecules within the Cytolytic Granules (Table VI)

i. Proteoglycans Proteoglycans of the chondroitin sulfate-A family are synthesized and remain cell associated within CL (MacDermott *et al.*, 1985; Stevens *et al.*, 1989). Both NK (Parmley *et al.*, 1985; Stevens *et al.*, 1987) and CTL (Young *et al.*, 1987) are known to store and secrete proteoglycans along with granule-bound perforin and granzymes (Schmidt *et al.*, 1985). The functions of proteoglycans have not been definitively identified, but they are acidic molecules, and their strongly negative charge at neutral pH

TABLE VI

Molecules Identified in Cytolytic Granules, Other Than Perforin and Granzymes[a]

Molecule	Putative function	Reference
Chondroitin sulfate proteoglycans	Inactivate granzymes	Stevens *et al.* (1987, 1989)
Calreticulin	Complexes free calcium	Burns *et al.* (1992)
DPP1 (cathepsin C)	Granzyme activation	McGuire *et al.* (1993)
TIA-1, TIA-R	Stress monitor, mRNA binding	Anderson *et al.* (1990, 1997)
Leukalexin	TNF-like cytotoxin	Liu *et al.* (1987)
Mannose-6-phosphate receptor	Granzyme targeting	Burkhardt *et al.* (1990); Peters *et al.* (1991); Griffiths and Isaaz (1993)
Lamp-1, lamp-2, CD63	Lysosomal constituent	Peters *et al.* (1991)
Arylsulatase, cathepsin D, β-glucuronidase, β-hexosamidase	Lysosomal enzyme	Tschopp and Nabholz (1990)
Leukophysin	Granule trafficking	Abdelhaleem *et al.* (1996)

[a] Adapted from Trapani and Smyth (1993).

may allow binding to the basic granzymes, thus preventing intragranule proteolysis of other constituents, including perforin. As stated above, proteoglycans may also play a part in the processing and packaging of granzymes through the Golgi. Chondroitin sulfate is capable of inhibiting perforin, and it may afford protection to the CTL/NK membrane following degranulation.

ii. Dipeptidylpeptidase 1 DPP1 (cathepsin C), a thiol protease, is the major processing enzyme responsible for the activation of myleoid and lymphoid granule serine proteases (McGuire *et al.,* 1993; Brown *et al.,* 1993). DPP1 activity is colocalized with granzyme A within granules and coexocytosed with it upon CD3 cross-linking of effector T cells (Brown *et al.,* 1993).

iii. TIA-1 Anderson and colleagues (1990; Kawakami *et al.,* 1992) first identified TIA-1 as the antigen recognized by a mAb, 2G9. cDNA clones encoding 40-kDa TIA-1 (p40 TIA-1) were isolated by expression screening of a λgt11 cDNA library and were found to encode a putative RNA-binding protein. The major granule-associated form has a molecular mass of 15 kDa and may be derived from the larger form by proteolytic cleavage. It may be targeted to the cytolytic granules by a lysosomal targeting sequence at the carboxy terminus. Because of its ability to bind nucleic acid, TIA-1 was for some time a candidate to induce DNA fragmentation in target cells. Indeed, purified TIA-1 was shown to be capable of inducing DNA fragmentation in digitonin-permeabilized target cells, but not in intact cells (Tian *et al.,* 1991). However, more recent data suggest that although TIA-1 may be granule associated, it is not normally exocytosed upon CL degranulation. Furthermore, its expression is not confined to CL, and TIA is also found in brain, spleen, and testis (Anderson *et al.,* 1997). TIA-1 is a modular protein with several amino-terminal RNA-binding and carboxy-terminal protein-interactive domains. It is now considered unlikely that TIA-1 plays a specific role in CL-mediated apoptosis, and its physiological role may be as a generic monitor of cellular stress. Under thermal and other environmental stresses, TIA-1 may aggregate with ribosomes on the endoplasmic reticulum to form "stress bodies," where its role may be to bind to and regulate the translation of key mRNA species involved in cell shutdown and apoptosis induction (Anderson *et al.,* 1997). This may explain the ability of TIA-1 to induce apoptosis when artificially introduced or transiently expressed in some cells.

iv. Leukalexin This molecule was first purified from the granules of mouse CTL and shown to be a 50-kDa protein with immunological cross-reactivity with TNFα (Liu *et al.,* 1987). Leukalexin was capable of inducing a slow, calcium-independent death in some cell types. There have been no further reports on this molecule since its initial description. An analogous

cytokine-like molecule antigenically unrelated to TNFα has also been described (Green *et al.*, 1986).

v. Granulysin A cDNA clone designated 519 was initially described during attempts to identify genes expressed specifically in the later stages of T cell activation (Jongstra *et al.*, 1987). The encoded protein, granulysin, is colocalized with granzymes and perforin in a subset of lytic granules and is exocytosed with them. On Percoll density gradients, lighter density cytolytic granules contain a 9-kDa form of granulysin that may be derived by processing of a 15-kDa form also present in lower density granules (Krensky *et al.*, 1997). A recombinant from of granulysin is cytolytic against tumor target cells at micromolar concentrations. Granulysin is structurally and functionally related to NK lysin, a molecule isolated from porcine intestine and shown to be microbicidal and tumoricidal. Both granulysin and NK lysin are related to the saposin family of membranolytic molecules. Neither is structurally related to perforin, and it is not yet known whether granulysin can augment the apoptotic actions of perforin and granzymes (Krensky *et al.*, 1997).

vi. Calreticulin This molecule has been isolated from lytic granules (Dupuis *et al.*, 1993; Bleackley *et al.*, 1995) and is thought to keep much of the calcium within cytolytic granules tightly chelated, to prevent premature activation of perforin. Upon exocytosis, dissociation of the complex may occur in the presence of higher calcium concentrations, allowing perforin to act on the target membrane.

vii. Lysosomal Enzymes The lysosomal proteins carboxypeptidase A, cathepsin D, arylsulfatase, β-glucuronidase, and β-hexosamidase have also been described in cytolytic granules (Millard *et al.*, 1984; Peters *et al.*, 1989; Tschopp and Nabholz, 1990).

C. The FasL/Fas Mechanism

1. Two Mechanisms of CL-Mediated Cytolysis

We have seen that, in addition to granule exocytosis, some CL, particularly $CD8^+$ CTL and $CD4^+$ Th1 CTLs, can express FasL to engage cell surface Fas and induce programmed cell death in the target cell. By contrast, the Th2 subset of $CD4^+$ T cells have been shown utilize the granule exocytosis mechanism when they display cytotoxicity (Lancki *et al.*, 1991). Also in accord with later studies, this group found that Th1 cells utilize a perforin-independent pathway, and several groups showed this to be the FasL pathway (Ju *et al.*, 1994; Stalder *et al.*, 1994). More recently, primary *in vitro* primed allospecific $CD4^+$ T cells were shown to kill through granule exo-

TABLE VII
Cytotoxic Mechanisms Used by Different Lymphocyte Subsets[a]

Lymphocyte subset	Cytotoxic mechanism	
	Perforin/granzyme	FasL/Fas
$CD8^+$ CTL	+	+
NK	+	−
$CD4^+$ Th1	−	+
$CD4^+$ Th2	+	−

[a] Adapted from Henkart (1994).

cytosis (Table VII). Killing through FasL presupposes the presence of Fas on the target membrane and a functional signaling pathway (see below), and like other members of the TNF receptor family, cross-linking (trimerization) of surface Fas is required for the subsequent transmissal of the death signal. Like its granule-based counterpart, FasL-mediated killing is rapid and occurs in the absence of protein or RNA synthesis; however, killing through FasL/Fas does not require calcium ions (Rouvier *et al.*, 1993). FasL expression on the surface of CTL can be induced during T cell activation (Suda and Nagata, 1994) and cytolytic activity can be blocked by soluble Fas-Fc (Ju *et al.*, 1994; Stalder *et al.*, 1994). More recently, the inference that FasL plays a role in cell death has been strongly supported by the clarification of the molecular mechanisms underlying the mouse *gld* and *lpr* mutations. The lymphoproliferative and autoimmune sequelae characteristic of both mutations are very similar, yet they map to different chromosomes. Two groups (Lynch *et al.*, 1994; Takahashi *et al.*, 1994) recently showed that *gld* is due to a point mutation within the cytoplasmic domain of the FasL molecule that results in ineffective transmission of the death signal. Unlike their wild-type counterparts, activated T cells from *gld* mice cannot kill target cells that express Fas (Hanabuchi *et al.*, 1994). By contrast, the insertion of a transposon in the Fas gene of *lpr* mice leads to a marked reduction (though not a complete absence) in expression of Fas in thymocytes and splenocytes and decreased sensitivity to anti-Fas antibodies (Watanabe-Fukunaga *et al.*, 1992). The residual levels of Fas expression in *lpr* mice account for the apparent normality of intrathymic clonal deletion in these animals.

2. Signaling through the Fas Receptor

The interaction between FasL and Fas is just one example of the growth-modulatory effects of the ever-broadening NGF/TNF family of receptor/

ligand pairs. Other members include type 1 and 2 receptors (TNFR1 and TNFR2) for TNF, NGF and its receptor NGF-R, CD27/CD27L, CD40/CD40L, CD30/CD30L, OX40/OX40L, 4-1BB/4-1BBL, and the more recently described receptors Apo-3, DR3, and WSL (reviewed in Nagata and Golstein, 1995). Cell killing by monocytes, like CL, involves a receptor/ligand pair from this family (TNF and the type 1 or p55 TNFR), indicative of the close evolutionary relationship between effector cytolytic responses. Defects of this mechanism result in normal T cell maturation and selection, but a defective response to certain pathogens (Pfeffer *et al.,* 1993). Unlike the majority of family members which are growth factors, FasL and TNF rapidly induce cell death and both mechanisms appear to recruit similar and overlapping signaling pathways.

Over the past 2 years, there has been an explosion of information regarding the signal transduction mechanisms that follow on from receptor ligation. Apoptosis can be induced in leukemic cells by high affinity antibodies to Fas, but not by $F(ab)'_2$ fragments, indicating that receptor aggregation is indispensible for signal transduction and is probably the principal function of interaction with the ligand (Krammer *et al.,* 1994; Cleveland and Ihle, 1995). Cocrystallization studies indicate that ligation of TNFR1 (Banner *et al.,* 1993) results in trimerization of the receptor. For both TNFR1 and Fas, there follows recruitment of a molecular signaling complex from components constitutively expressed within the cytoplasm (Cleveland and Ihle, 1995; Kischkel *et al.,* 1997). Using the yeast two-hybrid system, three structurally related proteins have been identified which are capable of binding to the cytoplasmic domains of Fas and/or TNFR1. The two receptors share a homologous cytoplasmic domain termed the death domain (Itoh and Nagata, 1993), which is necessary for their signaling function (Tartaglia *et al.,* 1993). The death domain is structurally similar to the *Drosophila* protein reaper, a 65-residue cytoplasmic polypeptide expressed in cells immediately prior to apoptosis (White *et al.,* 1994). The protein FADD (Fas-associated protein with a death domain) (Boldin *et al.,* 1995), also known as MORT-1 (Chinnaiyan *et al.,* 1995), can bind solely to Fas, while the analogous protein TRADD (TNFR1-related death domain protein) binds to TNFR1 (Hsu *et al.,* 1995), and receptor-interacting protein binds to both receptors (Varfolomeev *et al.,* 1996; Hsu *et al.,* 1996). All three proteins utilize their analogous death domain homology (DDH) regions to bind to the receptors.

A dominant negative form of FADD was able to block both Fas- and TNF-induced cell death, indicating that FADD is common to both signaling pathways (Chinnaiyan *et al.,* 1996). To account for this observation, it has been proposed that TRADD mediates the interaction of TNFR1 with FADD and downstream signaling events (Muzio *et al.,* 1996; Boldin *et al.,* 1996). In addition to its carboxy-terminal DDH regions, FADD encodes

an amino-terminal death effector domain (DED) which enables attachment of the most distal membrane-associated components of the death-inducing signaling complex (DISC). In normal circumstances, recruitment of latent cytoplasmic DISC proteins occurs extremely rapidly, within less than 1 s of Fas oligomerization (Kischkel *et al.,* 1997). FADD mutants that lack the DED domain act as dominant negatives by binding to Fas and inhibiting the addition of FLICE (FADD-like ICE) [Muzio *et al.,* 1996; also known as MACH (Boldin *et al.,* 1996)] or caspase-8 to the DISC. By contrast, the DED of FADD is independently capable of triggering apoptosis (Chinnaiyan *et al.,* 1995). Like FADD, pro-FLICE is a heterobifunctional molecule, consisting of two amino-terminal DEDs, one of which can interact with the DED of FADD, and a carboxy-terminal ICE-like cysteine protease domain (Muzio *et al.,* 1996; Boldin *et al.,* 1996). The function of the second DED of FLICE is probably to engage the first DED of FLICE in pro-FLICE, thus retaining the molecule in a catalytically inactive configuration until binding to the DISC has occurred. Binding of pro-FLICE to the remaining DISC proteins results in autocatalytic cleavage of the amino-terminal pro domain that includes the two DEDs and liberation of active FLICE from the DISC. FLICE is thus the critical ICE-like protease that sits at the "apex" of the cytoplasmic cysteine protease (caspase) cascade, conveying the death signal from the cell's membrane to its interior.

It should also be noted that in addition to domains that are conducive to apoptosis, Fas contains a negative regulatory element at its extreme carboxy terminus (Itoh and Nagata, 1993). This domain is no larger than 15 residues and has no equivalent in TNFR1. It is able to associate with a protein tyrosine phosphatase, PTP-BAS, that was originally identified in basophils (Maekawa *et al.,* 1994). Cells that are resistent to FasL and yet express Fas also express PTP-BAS, and enforced expression of the phosphatase in apoptosis prone cells confers resistance (Maekawa *et al.,* 1994).

3. The ICE-like Cytoplasmic Protease Cascade and Apoptosis

As indicated above, the net effect of recruitment of the proteins comprising the DISC complex to the inner leaflet of the cell membrane is the liberation into the cytoplasm of proteolytically active FLICE. It is postulated that FLICE then activates downstream proteases to bring about the characteristic degradative changes of apoptosis. Initial data implicating activated caspases as apoptotic effector molecules have arisen

1. by analogy to ced-3;
2. through the demonstration that specific protease inhibitors could block cell death. The cytokine response modifier A (crmA; Ray *et al.,* 1992)

protein of cowpox virus was first identified as blocking IL-1β production by binding to and inhibiting ICE, but it also blocks apoptosis induced through ICE (Gagliardini *et al.,* 1994), Fas (Tewari and Dixit, 1995; Strasser *et al.,* 1995), and TNF (Tewari and Dixit, 1995; Miura *et al.,* 1995). Peptidyl inhibitors of caspases such as tyr-val-ala-asp-CMK, asp-glu-val-asp-CMK, and *z*-val-ala-asp-FMK are potent inhibitors of many types of apoptosis, suggesting that caspase activation is a common feature of diverse forms of apoptosis (Zhivotovsky *et al.,* 1995; Sarin *et al.,* 1997a,b). Other apoptotic inhibitors are discussed below. Interestingly, ablation of ICE activity in the intact animal by targeted gene disruption (Li *et al.,* 1995) does not cause a gross disturbance of apoptosis. The phenotype of these mice is essentially normal except for resistance to Fas-mediated apoptosis in thymocytes and the predictable loss of active IL-1, suggesting a functional redundancy in caspases. Other caspases seem to be indispensible for homeostasis of specific organs, as evidenced by the fatal macrocephaly seen in CPP32 gene knock-out mice (Kuida *et al.,* 1996). Again, these mice have not been found to be immunologically compromised; and

3. by showing that overexpression of the ICE-like proteases can induce apoptosis (Miura *et al.,* 1993; Munday *et al.,* 1995) and that antisense expression inhibits it (Kumar, 1995).

4. Cleavage of Target Proteins by Activated Caspases Contributes to Apoptotic Morphology

A surprising early finding was that apoptotic cellular involution is not accompanied by random proteolysis, and the vast majority of cellular proteins remain intact until very late in the process (Kumar and Lavin, 1996). Nevertheless, certain specific structural and catalytic proteins are known to be cleaved early in apoptosis. The first to be described was the DNA repair enzyme poly(ADP-ribose) polymerase (PARP; Kaufmann, 1989; Kaufmann *et al.,* 1993). PARP can be cleaved by several of the caspases, including CPP32 (Tewari *et al.,* 1995a; Lazebnik *et al.,* 1994; Nicholson *et al.,* 1995), and a tetrapeptide corresponding to the cleavage site of PARP (asp-glu-val-asp*-gly, with the asterisk indicating the cleavage point) is a potent inhibitor of CPP32 (Nicholson *et al.,* 1995). PARP can also be cleaved by Mch3 (Fernandes-Alnemri *et al.,* 1995b), Mch2, and much less efficiently by ICE (Gu *et al.,* 1995). Although PARP appears to play a role in DNA maintenance and suppresses nuclease activity, its cleavage is not a *sine qua non* for apoptosis, as mice with a targeted disruption of the PARP gene have normal apoptosis (Wang *et al.,* 1995). ICE-like proteases also cleave the cell cycle regulatory and antiapoptotic protein pRb (Janicke *et al.,* 1996). Other nuclear structures known to be cleaved by activated caspases include the catalytic subunit of DNA-dependent protein kinase (DNA-

PKcs; Casciola-Rosen *et al.*, 1995; Song *et al.*, 1996), the sterol regulatory element-binding proteins 1 and 2 (Wang *et al.*, 1996), and nuclear lamins, cleaved by Mch2 (Lazebnik *et al.*, 1995). ICE is also able to cleave actin, thereby reducing its DNase I binding activity (Kayalar *et al.*, 1996), U1-associated 70-kDa protein (Casciola-Rosen *et al.*, 1996), and D4-GDI (Na *et al.*, 1996)

Importantly some caspases are also able to activate others in a hierarchical manner, thus amplifying the apoptotic cascade (Fig. 1). Some caspases including ICE are autocatalytic and others such as CPP32 contribute to their own processing (Fernandes-Alnemri *et al.*, 1996). For example, pro-CPP32 can initially be cleaved at asp175 by multiple proteases (including granzyme B) and then autocatalyzes further processing at asp9 and asp28. Pro-NEDD-2 can be activated by ICE (Harvey *et al.*, 1996) and Mch4, which like FLICE (Mch5) has two DED elements in its pro domain and can activate pro-CPP32 and pro-Mch3 (Fernandes-Alnemri *et al.*, 1995a). At least three activated caspases are generated in the cytoplasm that are capable of cleaving nuclear PARP and lamins: CPP32, Mch3, and Mch2. However, activated CPP32 does not accumulate within the nucleus (D. A. Jans and J. A. Trapani, unpublished observations) and nuclear accumula-

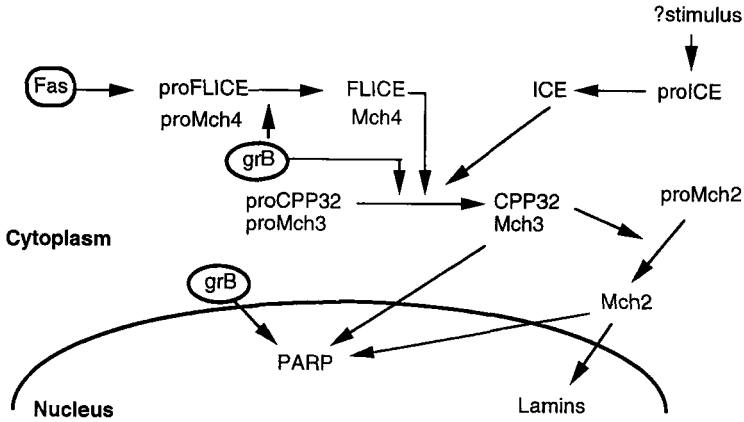

FIG. 1 A putative hierarchy of caspase specificities, and augmentation by granzyme B. Fas ligation and recruitment of FADD and other DISC proteins result in the activation of active FLICE (MACH) and Mch4. FLICE and possibly ICE activation result in the generation of at least three caspases known to cleave intranuclear target molecules exemplified by PARP and structural lamins. Granzyme B can activate the pathway at several steps, including at the apex of the cascade through cleave of pro-FLICE and pro-Mch4, and by cleaving pro-CPP32. In addition, in the absence of caspase inhibitors, granzyme B can target the nucleus directly, where it is likely to contribute to apoptotic morphology. The caspases and granzyme B also target key substrates in the cytoplasm and possibly at the cell membrane to induce cytoplasmic apoptotic changes that kill the cell independent of nuclear collapse (see text).

tion has not been reported for Mch2 and Mch3. Indeed, there is recent evidence that CPP32 may activate another cytoplasmic molecule, termed DFF (for DNA fragmentation factor), a heterodimeric factor composed of 40- and 45-kDa subunits that is responsible for DNA fragmentation (Liu *et al.,* 1997), suggesting that most of the nuclear events associated with CPP32 proteolysis may be indirect.

III. Molecular Pathology of CL-Mediated Apoptosis

A. Molecular Basis for the Synergy between Perforin and Granzyme B

1. Entry of Granzyme B into the Cell

Granzyme B does not induce apoptosis when cells are exposed to it in the absence of perforin (Shi *et al.,* 1992a,b; Jans *et al.,* 1996; Trapani *et al.,* 1997; Sutton *et al.,* 1997). Nevertheless, the requirement for perforin is not simply to provide a pore through which exogenous apoptotic agents such as granzyme B can diffuse into the cytoplasm. The minimal amounts of perforin required to induce apoptosis are insufficient to cause appreciable leakage of markers such as ^{51}Cr from the cells when used alone (Shi *et al.,* 1992a). Furthermore, we have tried to replace the function of perforin with other pore-forming/membrane-disruptive agents such as mild detergent (Shi *et al.,* 1997) and complement (J. A. Trapani, unpublished observations), but none of these agents can functionally replace perforin's collaborative role with granzyme B. In some of these experiments, even severe membrane disruption (>50% specific ^{51}Cr release, sufficient to permit macromolecules much larger than granzyme B access to the cytoplasm) was insufficient for apoptosis induction by granzyme B. The view that perforin is not necessary for internalization of granzyme B is supported by studies showing that it can cross the plasma membrane independent of perforin (Shi *et al.,* 1997; Trapani *et al.,* 1997; Froelich *et al.,* 1996b). In one study, the amount of granzyme B entering Yac-1 target cells in the absence of perforin was almost equivalent to that in its presence (Shi *et al.,* 1997), while another study demonstrated a significantly increased uptake into the cytoplasm when perforin was also added to the cells (Trapani *et al.,* 1997). Furthermore, the increased uptake of granzyme B in the presence of perforin was specific, as the same quantity of perforin had no effect on the uptake of a small, freely diffusible molecule such as 20-kDa dextran into the cell (Trapani *et al.,* 1997; Jans *et al.,* 1996).

2. Nuclear Targeting of Granzymes

Importantly, these studies also demonstrated an obvious redistribution of granzyme B from the cytoplasm into the nucleus when perforin was present, and nuclear accumulation correlated precisely with apoptosis (Trapani *et al.*, 1997). Studies performed with purified granzyme A also indicated that this granzyme undergoes nuclear localization very similar to that in granzyme B (D. A. Jans and J. A. Trapani, unpublished), whereas other serine proteases such as chymotrypsin do not accumulate in the nucleus (Trapani *et al.*, 1996). The kinetics of nuclear localization were considerably slower for granzyme A than those for granzyme B, consistent with the reduced DNA fragmentation observed with tryptase- rather than aspase-induced cell death (Shi *et al.*, 1992b; Shiver *et al.*, 1992). Entry of granzyme B into the nucleus preceded the onset of apoptosis as judged by (i) the impermeability of the nuclear membrane to 70-kDa dextran preloaded into the cytoplasm, and (ii) because the onset of DNA fragmentation and annexin V binding lagged well behind the extremely rapid (<2 min) nuclear targeting of granzyme B. This suggested that penetration into the nucleus preceded and was not simply a consequence of nuclear membrane disruption during apoptosis. The mechanism of granzyme B entry into the nucleus is not completely understood. Proteins smaller than ~45 kDa are normally able to enter the nucleus by diffusion (Jans *et al.*, 1991; Ymer and Jans, 1996). Some proteins of this size and all larger nuclear proteins also utilize a basic stretch of amino acids (the nuclear localization signal) that enables active transport across the nuclear pore complex. Granzyme B can accumulate rapidly in the nucleoplasm and nucleoli of artificially permeabilized hepatoma cells (without inducing apoptosis) (Jans *et al.*, 1996; Trapani *et al.*, 1994). This uptake is energy independent, requires a carrier molecule presumed to be a cytosolic protein, and is not dependent on proteolysis by granzyme B, as it still occurs in the presence of tripeptide granzyme B inhibitors (Jans *et al.*, 1996). Nucleolin, a protein found within both the cytoplasm and the nucleus, is bound and cleaved by granzyme A (Pasternack *et al.*, 1991); however, anti-nucleolin antibodies did not inhibit nuclear access of either granzyme A or granzyme B (Jans *et al.*, 1997). Neither granzyme A (D. A. Jans and J. A. Trapani, unpublished) nor granzyme B (Trapani *et al.*, 1996) can directly disrupt the integrity of the nuclear membrane, whereas prolonged incubation with chymotrypsin clearly can (D. A. Jans, unpublished observations).

3. Nature of the Perforin Signal

The above studies indicated that while perforin is essential for apoptosis, it is an oversimplification to see its role as as a passive transmembrane

conduit. Perforin may therefore exert its effect by alternative means. First, it may be possible for perforin to generate a membrane signal in its own right. However, no receptor has been found for perforin other than lipid molecules with phosphorylcholine headgroups (Tschopp *et al.,* 1989) and at the present time there is no report of phosphorylation or other membrane signaling events being generated following perforin's attachment to the cell. A further possibility is that perforin provides release of granzymes into the cytoplasm from some shielded compartment such as an endosome. A model that addresses this possibility is that advanced recently by Froelich *et al.* (1996b). These investigators showed evidence of a saturable granzyme B cell surface receptor that enabled it to enter the cell, presumably by endocytosis. The identity of such a receptor is unclear; however, the same group demonstrated that granzyme A could induce IL-6 and IL-8 production by interacting with cell surface binding sites (Sower *et al.,* 1996a,b). The cells exposed to granzyme B alone remained viable; however, if granzyme B was introduced with a noncytopathic replication-deficient adenovirus, they underwent rapid apoptosis. A key mechanism of adenovirus pathogenicity is that following its endocytosis, it can escape endosomes into the cytoplasm. This attribute has previously been utilized by researchers to introduce proteins into cells (Seth, 1994). Froelich hypothesizes that escape of granzyme B into the cytoplasm due to adenovirus allows it to access key caspase substrates and that this mimics the role normally played by perforin. That is, perforin's main role is to disrupt endocytic vesicles, and it uses a form of viral mimicry to achieve this end. The remaining objection to this intriguing possibility is that entry of perforin into the cell has never been definitively demonstrated.

4. Effects of Granzyme B/Perforin on the Cell Cycle

One hypothesis regarding the actions of perforin and granzymes in eliciting apoptosis has centered on the possibility that granzyme B can disrupt normal progression through the cell cycle, leading to an inappropriate and untimely entry into mitosis, i.e., death by "mitotic catastrophe" similar to that observed in yeasts and some mammalian cells (Krek and Nigg, 1991; Heald *et al.,* 1993). Cells undergoing apoptosis in response to granzyme B and perforin were noted by Greenberg and colleagues to rapidly upregulate the activity of the cyclin-dependent kinase cdc2 to the same levels as those in cells that were blocked in G_2/M with the chemical agent nocodazole (Shi *et al.,* 1994). Furthermore, the expression of cdc2 kinase activity was necessary for chromatin condensation and DNA fragmentation in response to granzyme B. Normally, cdc2 kinase activity is regulated by means of its binding to cyclins A and B, resulting in its translocation into and out of the nucleus at appropriate times in the cell cycle, and by its phosphorylation/

dephosphorylation in response to the kinase Wee 1 [which inactivates it by phosphorylation at tyr15 (Parker *et al.,* 1993)] and the phosphatase cdc25 [which dephosphorylates the same tyr residue (Gould *et al.,* 1990)]. Overexpression of Wee 1 kinase was able to rescue cells from granzyme B/perforin-induced apoptosis (Chen *et al.,* 1995). Furthermore, cells arrested in G_0 do not express cyclin-dependent kinases and are less susceptible to CTL (Nishioka and Welsh, 1994), whereas Jurkat cells purified from all other stages of the cell cycle were equally susceptible to granzyme B and perforin (Shi *et al.,* 1996). Although supported by a considerable amount of experimental evidence, this hypothesis has remained controversial. There are instances where cdc2 kinase is not required in other types of apoptosis (Freeman *et al.,* 1994), raising the unlikely scenario that this mechanism may be peculiar to CL-induced apoptosis. Apoptosis of transformed rat embryo fibroblasts was not accompanied by dephosphorylation of tyr15 (Oberhammer *et al.,* 1994). Furthermore, temperature-sensitive mutants of the cell line FT210 continued to be partially sensitive to granzyme B, even when cdc2 kinase activity was reduced to very low levels (Shi *et al.,* 1994). Similar findings were reported by Lazebnik *et al.* (1993) who found no effect on the kinetics or total extent of apoptosis induction despite removal of >90% of cdc2 kinase activity. At a minimum this argues that cdc2-independent pathways of granzyme B/perforin-mediated killing must also exist.

5. Granzyme B Is an Aspase That Can Activate and Augment the Caspase Cascade

The ability of granzyme B to cleave key substrates at asp residues was first suggested by Vaux *et al.* (1994) as a means by which CTL might activate the death cascade in targeted cells. We have seen that the CL of mice deficient in granzyme B induce apoptosis with markedly slowed kinetics compared with wild-type mice (Heusel *et al.,* 1994); nevertheless, this defect can be corrected by longer incubation times, suggesting that alternative mechanisms are present within CL granules that can overcome granzyme B deficiency. The ability of granzyme A to induce apoptosis in several independent experimental systems in the absence of granzyme B (Shiver *et al.,* 1992; Shi *et al.,* 1992a,b) argues for its ability to complement the actions of granzyme B or even overcome its absence. Granzyme A has been shown to have ICE-like activity, in that it can produce active IL-1β by cleaving pro-IL-1 at a tryptase site adjacent to the asp residue usually cleaved by ICE (Irmler *et al.,* 1995). However, granzyme A has not been demonstrated to activate any of the caspases, and the mechanism by which apoptosis is brought about by granzyme A is uncertain. Indeed, a caspase inhibitor that blocked granzyme B-mediated apoptosis had no effect on cell death caused by granzyme A (Anel *et al.,* 1997). Nevertheless, granzyme

A-deficient mice have no obvious defect of apoptosis induction (Ebnet *et al.,* 1995). By contrast with granzyme A, there is now strong evidence that granzyme B can activate many of the pro caspases (Fig. 1). *In vitro,* granzyme B has been shown to activate pro-CPP32 (Darmon *et al.,* 1995; Fernandes-Alnemri *et al.,* 1996; Martin *et al.,* 1996) and pro-Nedd-2 (Harvey *et al.,* 1996), but it cannot cleave pro-ICE (Darmon *et al.,* 1994). It has not been adequately demonstrated whether granzyme B acts directly on pro-CPP32 *in vivo.* Activation of CPP32 by CL appears to be granzyme B dependent, but the effect might be indirect, as granzyme B is also capable of activating upstream caspases including pro-FLICE (Mch5) and pro-Mch4, both of which can also cleave CPP32 (Fernandes-Alnemri *et al.,* 1996). Granzyme B's ability to activate FLICE should in theory be sufficient to activate the whole cascade, but *in vitro* data suggest that granzyme B can cleave multiple caspases, thus amplifying the cascade both proximally through FLICE and Mch4 and more distally through CPP32, Mch3 (Fernandes-Alnemri *et al.,* 1996), Mch2 and Mch6 (Srinivasula *et al.,* 1996a), and CMH-1/ICE-LAP3 (Gu *et al.,* 1996). The cleavage of pro-Mch6 is at a site distinct from that utilized by CPP32 (Srinivasula *et al.,* 1996a) (Table II). The overall result is the generation of multiple active proteases that can cleave nuclear structures such as PARP and lamins in addition to cytoplasmic substrates. Granzyme B does not appear to activate Mch2 directly, although this protease is thought to act on lamins directly (Fernandes-Alnemri *et al.,* 1995b). Darmon and colleagues (1995) used tetrapeptide inhibitors that are relatively specific for caspase subfamilies to dissect the roles of the different proteases in DNA fragmentation and cell membrane damage. Ac-asp-glu-val-asp-CHO, which inhibits CPP32 and related protease Mch3 (Nicholson *et al.,* 1995), had no effect on ^{51}Cr release from target cells killed by a granule-dependent mechanism but dramatically reduced DNA fragmentation from the same cells. In contrast, Ac-tyr-val-ala-asp-CHO, which inhibits ICE (Thornberry *et al.,* 1992) and related proteases but not CPP32 (Nicholson *et al.,* 1995), had no effect on DNA fragmentation. Furthermore, mice deficient in ICE have apparently normal apoptosis (Li *et al.,* 1995). This suggests that the CPP32 family of proteases is instrumental in eliciting nuclear damage, while the ICE-related proteases have little effect. This study, like that of Sarin *et al.* (1997a), suggested that neither group of caspases plays a significant role in the cell membrane damage resulting from granule-induced apoptosis. *In vitro* evidence suggests that granzyme B may also be capable of cleaving target proteins such as PARP (Froelich *et al.,* 1996a) and DNA-PKcs (Song *et al.,* 1996) directly, but in each case the cleavage sites are different from those used by the caspases. Unlike the caspases, there is good evidence that granzyme B is able to reach the nucleus in high concentrations and

thus may be able to access and cleave nuclear substrates directly (Trapani *et al.,* 1996; Jans *et al.,* 1996; Pinkoski *et al.,* 1996).

B. Naturally Occurring Inhibitors of Granzyme/Perforin and Fas/TNF-Mediated Apoptosis

1. Bcl-2/CED-9-like Inhibitors

The mammalian family of Bcl-2-like molecules includes both anti (Bcl-2, Bcl-X_L)- and pro (Bax, Bik, Bcl-X_S)-apoptotic members (Cory, 1995; Vaux *et al.,* 1992a). Bax forms heterodimers with Bcl-2 and opposes its activity, and the relative quantities of Bcl-2 and Bax within a cell can determine whether a cell will undergo apoptosis in response to a given stimulus. Ced-3-mediated cell death in nematodes is blocked by Ced-9, but in mammalian cells, Bcl-2 can block some, but not all, forms of apoptosis reliant on caspase activation. Itoh and Nagata (1993) showed partial inhibition of anti-Fas antibody-mediated killing of FDC-P1 mouse myeloid cells; however, Bcl-2 could not block Fas-mediated killing of lymphoid cells (Strasser *et al.,* 1995). Reports on the ability of Bcl-2 to block CL-mediated apoptosis have been inconsistent, and the end result may be a function of the target cell type (Sutton *et al.,* 1997). Some reports have indicated that Bcl-2 can protect against allogenic CTL attack (Schroter *et al.,* 1995), while others have suggested otherwise (Vaux *et al.,* 1992b; Sutton *et al.,* 1997). Chiu *et al.* (1995) found protection against granule- but not CD95-mediated attack. We recently demonstrated that although Bcl-2 was unable to block apoptosis induced by intact CL or isolated granules, it completely blocked cell death induced by isolated granzyme B and perforin (Sutton *et al.,* 1997). The implication is that cytolytic granules contain components that can bypass the Bcl-2-mediated block of granzyme B.

2. CrmA

This product of the cowpox virus (Ray *et al.,* 1992) is one of a number of poxvirus proteins that interfere with the host's ability to eliminate virus-infected cells. CrmA is an intracellular serpin (serine protease inhibitor) that, like other serpins, acts in a cognate manner as a pseudosubstrate for a given protease. The specificity of the interaction is dictated by the residue at the P_1 position of the inhibitory loop (an asp residue in CrmA), which mimics the natural substrate preference of the protease but locks it into an irreversible complex that can be identified on SDS–PAGE (Potempa *et al.,* 1994). Some serpins including CrmA can inhibit proteases of more than one subclass, as exemplified by CrmA's ability to block many cysteine

proteases, including ICE (Ray *et al.,* 1992; Haecker *et al.,* 1996), CPP32 (Tewari *et al.,* 1995a), and FLICE (Srinivasula *et al.,* 1996b) in addition to the serine protease granzyme B. The caspase-blocking activity of CrmA results in a block of apoptosis induced by growth factor (Gagliardini *et al.,* 1994) or serum (Wang *et al.,* 1994) withdrawal, TNF (Tewari *et al.,* 1995a), and Fas (Tewari *et al.,* 1995a; Enari *et al.,* 1995). CrmA's ability to block Fas is reflected in its inhibition of Fas-mediated CL-induced apoptosis (Tewari *et al.,* 1995b; Macen *et al.,* 1996). CrmA is also capable of blocking granzyme B's asp-ase activity *in vitro* (Quan *et al.,* 1995); however crmA's binding to granzyme B is at least 10-fold lower than that to ICE (J. Sun, P. I. Bird, and J. A. Trapani, unpublished results). CrmA does not block other granzymes, as its effect is restricted to proteases with asp-ase activity. CrmA expression in cell lines can block perforin/granzyme B-mediated apoptosis (Tewari *et al.,* 1995b; Macen *et al.,* 1996); however, the effect is far weaker than that for the Fas pathway. The physiological relevance of this observation is unclear, as target cells from CrmA-transgenic mice are as susceptible to attack by allogenic CTL or CTL granules as those of nontransgenic littermates (V. R. Sutton and J. A. Trapani, unpublished observations).

3. GBI (PI-9), a Naturally Occurring Inhibitor of Granzyme B Expressed by CL

A novel intracellular serpin was recently described which is expressed at high levels in cells with cytolytic capacity (Sun *et al.,* 1996). The sequence of this serpin was identical to another which had been cloned and designated PI-9 without assignment of a function (Sprecher *et al.,* 1995). The inhibitory loop was strongly related to that of crmA; however, the asp at the P_1 position of crmA was not conserved but was replaced with another acidic residue, glu (Sun *et al.,* 1996) (Fig. 2). Addition of recombinant PI-9 to

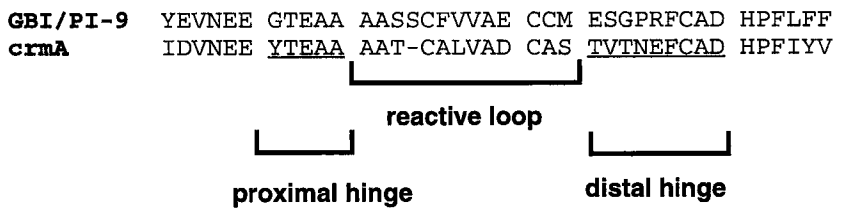

FIG. 2 The inhibitory loop regions of the serpins GBI/PI-9 and crmA. The single-letter amino acid code is used. The reactive site residues of the two serpins are optimally aligned and the P1 residue is marked with an asterisk. The flanking conserved hinge residues are underlined. Compiled from Sun *et al.* (1996) and Ray *et al.* (1992).

granzyme B resulted in a stable complex with an association constant that was within the range for physiologically significant serpin–protease interactions. Importantly, PI-9 was able to completely abrogate apoptosis when preincubated with granzyme B and perforin and so was given the alternative name of granzyme B inhibitor (GBI). Its expression in transfectant cell lines also affords protection against granule-mediated cell death (Bird *et al.,* 1998). Subcellular fractionation studies showed GBI to be a cytosolic protein that is not secreted and is absent from cytolytic granules (Sun *et al.,* 1996). It was hypothesized that GBI/PI-9 might inactivate misdirected granzyme B molecules following packaging or degranulation, thereby protecting the CL from inadvertant autolysis. It is not clear why GBI utilizes glu rather than asp at the P_1 position, as granzyme B cleaves synthetic substrates at asp far more efficiently than those at glu (Poe *et al.,* 1991). Indeed, substitution of the P_1 glu with asp does not result in increased binding to granzyme B (P. I. Bird, personal communication). As with many other serpins, the GBI residues adjacent to P_1 probably influence the shape of the substrate pocket with which interactions are possible. It is not yet known whether GBI can interact with any of the caspase proteins or block Fas-mediated cytolysis. The rodent equivalents of GBI have yet to be definitively identified, although it is clear that T mouse splenocytes synthesize a broad variety of endogenous serpins, and at least two of these molecules have inhibitory loops with key acidic residues with the potential to interact with granzyme B (Sun *et al.,* 1997).

4. Baculovirus p35

p35 is a baculovirus protein that exerts a strong inhibitory effect on caspase-dependent apoptosis in infected insect cells (Clem *et al.,* 1991; Clem and Miller, 1993). It has recently been shown to inhibit a broad variety of caspases, including ICE, Ced-3, Nedd2, and CPP32 (Xue and Horvitz, 1995; Bump *et al.,* 1995), and probably does so by forming a stable complex with the enzymes, akin to crmA. Cleavage of p35 at asp87 is essential for its inhibitory activity, both *in vitro* and in *C. elegans.* Sarin *et al.* (1997a) have demonstrated that, like the caspase inhibitor val-ala-asp-FMK, p35 can block both the nuclear and the cytoplasmic consequences of CL-mediated apoptosis through Fas, but it blocks only the nuclear consequences of granule-mediated cytolysis. p35 expression can also inhibit neuronal apoptosis due to factor withdrawal (Rabizadeh *et al.,* 1993) and TNF-induced cytolysis (Beidler *et al.,* 1995).

5. Other Inhibitors

There is a rapidly growing list of viral inhibitors of apoptosis; indeed, it is clear that the coevolution of viruses and their host cells has resulted in

every facet of recognition and apoptosis induction being inhibitable by viruses. Baculovirus IAP (inhibitor of apoptosis; Clem and Miller, 1994) is a broadly active serpin with a RING finger domain and homology to several mammalian proteins (Uren *et al.*, 1996; Liston *et al.*, 1996), including the product of the gene which is defective in the degenerative disease spinal mucular atrophy (Roy *et al.*, 1995). Two of the mammalian forms exert their effect by binding to the TNF receptor 2-associated proteins, TRAF 1 and TRAF 2 (Uren *et al.*, 1996), and are able to block both Fas- and TNFR-mediated cell death. The Epstein–Barr-encoded protein BHRF, African swine fever virus LMW5-H1, and herpesvirus samrai ORF16 are Bcl-2-like in structure, while the adenovirus E1B protein (White *et al.*, 1991) shows no similarity to Bcl-2 but can heterodimerize with Bcl-2-like molecules and inhibit their activity (Farrow *et al.*, 1995). Rabbitpox virus SPI-1 has low sequence similarity to SPI-2 (the equivalent of cowpox virus crmA) but probably also acts as a caspase inhibitor (Brooks *et al.*, 1995). Other viral proteins acting at the apex of the caspase cascade are the FLICE inhibitory proteins (FLIPs). These proteins encode two death effector domains that can interact with FADD to prevent attachment of FLICE to the DISC, thus preventing its subsequent activation (Thome *et al.*, 1997; Bertin *et al.*, 1997; Hu *et al.*, 1997). Mammalian homologs of the FLIPs also exist and probably have a role in regulating FLICE activation (J. Tschopp, personal communication). Viral proteins produced by herpes simplex can reduce deployment of FasL (Sieg *et al.*, 1995), while others can affect p53 ubiquitination and degradation (Cheng *et al.*, 1997). Another protein shown to interact with the death domains of Fas and TNFR1 and block signal transduction has been termed sentrin (Okura *et al.*, 1996).

C. Nuclear versus Cytoplasmic Targets of Apoptotic Proteases: Convergence of the Granzyme/Perforin and Fas Pathways

It is clear from the above discussion that the granule exocytosis mechanism functions at least partly through the same intracellular effector molecules as are activated by the FasL/Fas pathway. What, then distinguishes the two pathways? Superficially, it would seem to be a point of vulnerability that both mechanisms used by CL should converge so closely in the same biochemical pathway. Evidence is now emerging that the two mechanisms *are* biochemically distinguishable, in that unlike the Fas pathway, the granule exocytosis mechanism can kill cells independent of caspase-induced proteolysis. In one study, serine protease activity was required for both cell membrane damage and DNA fragmentation; however, addition of the serine protease inhibitor DCI 15 minutes after the initiation of the lytic

cycle resulted in abolition of DNA fragmentation without an effect on membrane damage (Helgason *et al.,* 1995). Sarin *et al.* (1997a) used synthetic peptide inhibitors to show that both nuclear and cytoplasmic phenomena of apoptosis mediated through Fas are dependent on caspase activation. They found that blocking all caspases (or just key capsases such as FLICE sitting at the apex of the cascade) can abrogate apoptosis through Fas. *Nuclear* apoptotic changes brought about through granule exocytosis were also blocked by these inhibitors, but cells exposed to this pathway still died by apoptosis, as manifested by *cytoplasmic* shutdown. Furthermore, these manifestations were not simply due to the unopposed actions of perforin acting alone on the cell membrane; rather, proteolysis (presumably by granzymes acting independent of caspases) was also required (Sarin *et al.,* 1997a,b).

Applying these observations hypothetically to cells infected with everyday virus infections, one would envisage the following scenarios. In the normal course of an infection with a "benign" virus, i.e., one *in*capable of blocking the caspase cascade, an infected cell could be killed by either or both pathways and dies a rapid death as a consequence of both nuclear and cytoplasmic apoptotic changes. In this scenario, nuclear degradation would be accomplished by the joint actions of both granzyme B and certain activated caspases, probably acting on different cleavage sites on targeted proteins (for example, as shown in Fig. 1). In the alternative scenario, a "less benign" virus might be able to completely block the caspase pathway (and therefore Fas-mediated apoptosis) to prolong its own survival (see below). In evolutionary terms, such a situation should probably arise relatively infrequently, as it is disadvantageous for a virus to kill its natural host. Rather, it is more clearly advantageous to *delay* apoptosis, thus facilitating its spread to uninfected cells, or to survive in a dormant or cryptic form until conditions are ripe for infection of fresh cells. Nevertheless, when confronting a serious infection, the CL must be capable of having the final say on the survival of an infected cell, and it does so by enlisting the granule exocytosis mechanism. Thus, if the caspase cascade is blocked in a cell, it still dies because of the cytoplasmic consequences of apoptosis. The means by which CTL granules achieve caspase-independent apoptosis is an important unresolved issue, one possibility being that granzymes can initiate apoptosis by targeting their own unique substrates in the cytoplasm (Fig. 3).

Although morphologically the most obvious part of the normal phenomenology of apoptosis, nuclear collapse is clearly not a *sine qua non* of apoptotic cell death. Indeed, apoptosis is not reliant on the presence of a nucleus, and cell death is seen in its absence (Schulze-Ostoff *et al.,* 1994; Nakajima *et al.,* 1995; Jacobson *et al.,* 1994). Nor is DNA fragmentation an invariable consequence of apoptosis. For example, mutant fibroblasts lacking endoge-

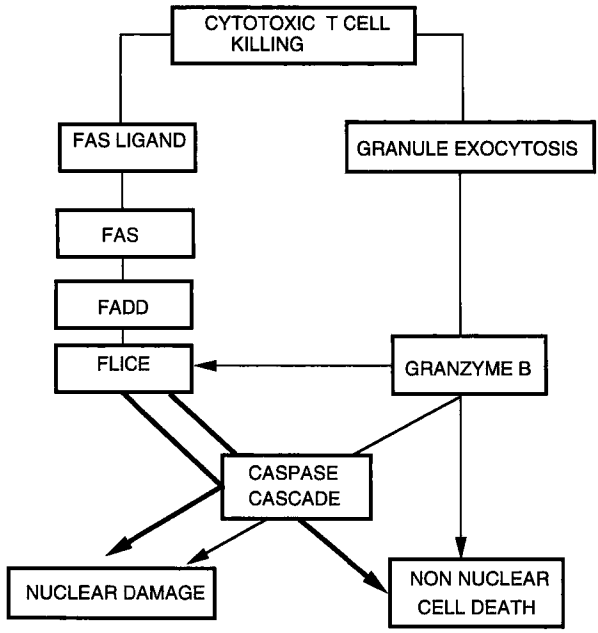

FIG. 3 A schematic representation of the relative dependence on caspase activation of FasL- and granzyme/perforin-mediated nuclear and cytoplasmic features of apoptotis. Inhibitor studies with the broadly active serpin, p35, and synthetic tripeptide fluoromethylketone derivatives (Sarin *et al.*, 1997a,b) suggest that both the nuclear and the cytoplasmic consequences of triggering through the Fas pathway are caspase dependent. The nuclear apoptotic changes observed with granzyme B and perforin (including nuclear targeting of granzyme) are also caspase dependent, but granule-mediated cytolysis can still proceed in a caspase-independent manner through cleavage of as yet unidentified cytoplasmic target molecules.

nous nucleases underwent apoptosis in response to CTL just as rapidly as their wild-type counterparts (Ucker *et al.*, 1992). It therefore follows that nuclear damage is likely to be downstream of "cytoplasmic death," and although many of the identified nuclear targets of caspases and granzyme B explain aspects of apoptotic morphology, there is a pressing need to define the cytoplasmic processes that underpin the entire mechanism. Some exciting recent findings have recently begun to address these issues. Two groups of investigators have recently shown that during apoptosis, the apoptotic protein Ced-4 can interact with Ced-3 and its mammalian homologues, ICE and FLICE (Wu *et al.*, 1997; Chinnaiyan *et al.*, 1997). Overexpression of Ced-4 in mammalian cells induced cell death, and further overexpression of Bcl-X_L in these cells blocked it, as did the addition of caspase inhibitors (Chinnaiyan *et al.*, 1997). The antiapoptotic protein Ced-9 can negatively regulate apoptosis by binding to Ced-4, and Ced-4 interacts with

Ced-3 or the mammalian equivalent proteins such as ICE and FLICE, which have large pro domains, but not with CPP32 or Mch2a, which have small pro domains. Thus, Ced-4 plays a pivotal role in regulating cell death and links pro-apoptotic proteases of the ICE family with inhibitory proteins of the Bcl-2 family, by its ability to directly interact with both families of molecules. Although the apoptotic mechanism of *C. elegans* is much simpler than that of mammals, it is highly likely that a mammalian equivalent of Ced-4 exists. By analogy with both the ICE and the Bcl-2 families, it is indeed possible that multiple Ced-4-like proteins may exist. Normally, Ced-4 is expressed in the cytoplasm; however, when Bcl-X_L is also expressed, the Ced-4/Bcl-X_L complex is relocated to mitochondrial membranes (Wu *et al.,* 1997). In so doing, Ced-9 appears to modify Ced-4, thereby preventing it from activating Ced-3 and dislocating upstream signaling events from caspase activation. Conversely, in the absence of Ced-9/Bcl-2, Ced-4 is free to activate the protease cascade.

It is likely that additional molecules may also link caspases and Bcl-2-like molecules, although how this relates to Ced-4 is unclear. Cytochrome *c* release from mitochondria is also required for caspase activation, and Bcl-2 appears to regulate the release of cytochrome *c* during apoptosis (Kluck *et al.,* 1997). Bcl-2 expression also reverses the loss of mitochondrial membrane potential which occurs soon after induction of apoptosis (Marchetti *et al.,* 1996), preventing the release of cytochrome *c* (Yang *et al.,* 1997). This Bcl-2 inhibitable process has been shown to be important for apoptosis induction in lymphocytes (Marchetti *et al.,* 1996). Loss of permeability leads to the release of AIF (apoptosis-inducing factor) from mitochondria into the cytosol (Susin *et al.,* 1996). AIF is itself a caspase-inhibitor-sensitive protease which can cause caspase activation and cell death (Kroemer *et al.,* 1997). Overexpression of Bcl-X_L, which forms ion channels in membranes (Minn *et al.,* 1997), has been shown to kill cells without the activation of caspases (Xiang *et al.,* 1996). It has been proposed that Bcl-X_L may mimic the effects of perforin in this regard (Golstein, 1997). In each case, a pore-forming protein may facilitate the release of another pro-apoptotic protein (granzyme B from endocytic vesicles in the case of perforin; AIF from mitochondria in the case of Bcl-X_L) from a membrane-bound intracellular compartment into the cytosol where the caspase cascade may be activated.

D. The Role of CL-Mediated Killing in Resistance to Virus and Other Intracellular Infections

Although it provides a means of detecting and eradicating intracellular pathogens, cell-mediated cytolysis is not the only means by which the

immune system controls virus infections. NK cells can limit the spread of early infection by limiting viral replication through secretion of IFNγ, and this can be a crucial while a cognate T cell response is generated (Welsh, 1981). In other situations, e.g., infection with vesicular stomatitis virus (VSV) or Semliki Forest virus (SFV), the production of neutralizing antibodies may be the crucial event in an effective defense (Zinkernagel and Rosenthal, 1981). The relative importance of these various mechanisms in the overall response to various viruses has been the subject of hot debate, as exemplified by the observations that recombinant vaccinia viruses that secrete IFNγ or TNF were considerably less virulent than their wild-type counterparts (Ramsay *et al.,* 1993) and, furthermore, that gene knockout mice lacking these T-cell-dependent cytokines were more susceptible to vaccinia (Muller *et al.,* 1994). We have seen above that many viruses have developed ways of subverting intracellular apoptotic pathways, especially those involving caspase signaling. Given the close evolutionary relationship between viruses and their hosts, it would seen likely that specific means of blocking perforin or granzyme function might have also arisen. Other than for the inhibitory effects of crmA on granzyme B and the observation that parainfluenza virus can downregulate the expression of grB mRNA in a selective manner in infected T cells (Sieg *et al.,* 1995), information is lacking in this area.

Kagi and colleagues (1995) examined the responses of perforin- and FasL-deficient mice to infection with noncytopathic LCMV and three cytophatic viruses, VSV, SFV, and vaccinia. They found that an intact perforin pathways was crucial for recovery from LCMV; however, neither the perforin nor the FasL pathway was crucial for recovery from the cytopathic viruses. In addition, perforin appears to be crucial for recovery from infections with the intracellular bacterial pathogen *Listeria monocytogenes* (Kagi *et al.,* 1994b). They hypothesized that contact-dependent cytotoxicity (through perforin) might only be crucial in noncytopathic infections, as the virus remains hidden within the cell for a considerable period after a display of viral antigens on the cell surface. On the other hand, in a cytopathic infection, CL-mediated cytolysis may proceed too slowly to efficiently counter spread of the virus, and the immune system may then rely on cytokines to limit replication and neutralizing antibodies to clear the residual infection. Notwithstanding these general "rules," it can be difficult to predict which type of response will be critical in any given infection, and it may even vary from organ to organ. Splenic $CD8^+$ T cells and NK cells contribute to recovery from mouse CMV infection in a perforin-dependent manner; however, in the liver, the predominant effector mechanism involves IFNγ secretion (Tay and Welsh, 1997). In immunocompromised human bone marrow transplant recipients, protection against CMV infection depended on $CD8^+$ CTL, and adoptive transfer of these cells from healthy donors

afforded protection from both asymptomatic viremia and pneumonia (Greenberg, 1997). Cytokines and the products of cell lysis can contribute to tissue inflammation and may also significantly contribute to pathology, perhaps in part by injuring bystander cells that do not harbor virus. CTL may therefore contribute to the pathology of chronic viral hepatitis due to both hepatitis B (HBV) and hepatitis C (HCV) viruses. In chronic HCV infection, FasL is expressed by liver-infiltrating T cells, some of which also appear to kill Fas^+ antigen-expressing hepatocytes (Ando *et al.,* 1997). An incomplete cytolytic response early in the course of infection may result in chronic hepatitis mediated by CTL, or, alternatively, the continuing death of hepatocytes may result in secondary inflammatory responses, leading to cycles of degeneration and regeneration culminating in cirrhosis.

E. Roles of Perforin and FasL in Immune Response to Tumors

The putative roles of both innate and acquired immune responses to tumor formation have long been a topic of debate (Karre *et al.,* 1986). Recent findings with perforin-deficient mice have provided definitive evidence that the perforin-dependent and -independent pathways contribute to immune surveillance. In one study (van den Broek *et al.,* 1995), NK cells, which in some models are of importance in protecting against tumors lacking MHC antigen expression (Wagtmann *et al.,* 1995; Karre *et al.,* 1986), were shown to control class I-negative RMA-S lymphoma formation in a perforin-dependent manner. In these studies, the inability of perforin-deficient NK cells to kill RMA-S cells *in vitro* correlated well with tumor growth *in vivo.* In a more extensive study using perforin-deficient and wild-type mice, tumors either were induced *de novo* by a variety of means including chemical carcinogens and oncogenic virus or were grown in the animals following administration of varying doses of syngeneic tumor cell lines (van den Broek *et al.,* 1996). Differences were found between the groups of mice, with respect to both rejection of syngeneic tumors and the genesis of new tumors. Most of the tumor lines were rejected 10- to 100-fold more efficiently by the perforin-expressing mice, and the difference was more marked following priming. Introduction of Fas by transfection into some of the cell lines resulted in somewhat improved tumor rejection, when compared with untansfected controls; however, the major contribution to tumor rejection was through the perforin pathway. Induction of tumors with methylcholanthrene was more rapid in the perforin-deficient mice; however, an alternative carcinogen, DMBA, produced similar numbers of skin papillomas in both sets of mice. Furthermore, the regression of Moloney murine sarcoma virus-induced tumors was slower in perforin-deficient mice. Overall, it was

concluded that perforin-mediated cytolysis is instrumental in protection against both the induction of new tumors and the immune response to established tumors. Further evidence of the role of CL is provided by the observation that, under selective pressure, tumors can reduce the expression of class I, class II, or costimulatory molecules by a variety of means to escape cytolysis (Uyttenhove *et al.,* 1983).

Disturbingly, it is now also becoming apparent that tumors can turn the tables on the immune system, expressing death molecules such FasL that may enable them to evade the immune response by granting them a form of "immune privilege" (Nagata, 1996). It was first reported that some NK cell leukemias and lymphomas can constitutively express FasL (Tanaka *et al.,* 1996), but the expression is also seen on nonhematological malignancies such as melanoma (Hahne *et al.,* 1996), colon carcinoma (O'Connell *et al.,* 1996), and hepatocellular carcinoma (Strand *et al.,* 1996). In one of these studies, the growth of FasL-expressing melanomas was significantly retarded in *lpr* mice whose T cells cannot express functional Fas. The strong implication is that the tumor can kill tumor-infiltrating inflammatory cells such as CL that express Fas. Concomitantly, the tumors can either downregulate their own expression of Fas or render themselves unable to signal their own apoptosis, thereby escaping the possibility of fratricidal (or even CL-induced) cell death. Perhaps even more disquieting is the revelation that some cancer therapies such as chemotherapy may induce the expression of FasL on tumor cells, thus facilitating destruction of antitumor lymphocytes (Strand *et al.,* 1996).

F. Roles of Perforin and FasL in Graft Rejection, Antigen Presentation, and Autoimmune Disease

Mice deficient in perforin demonstrate a reduced ability to mount an allogeneic mixed lymphocyte response *in vitro* (Kagi *et al.,* 1994a), and a variety of abnormalities in rejecting allogeneic and xenogeneic tissues and tumors. Perforin was not required for the rejection of allogeneic heart transplants across a complete H-2 mismatch, and the grafts were infiltrated with phenotypically similar populations of mononuclear cells irrespective of the presence of perforin. However, the granule exocytosis mechanism was important when the donor and recipient tissues were separated by a smaller difference, e.g., mismatch at just one class I antigen (Schulz *et al.,* 1995). In a study examining transplanted pancreatic islet tissues, neither the perforin nor the FasL pathways were found to be critical for graft rejection, as exemplified by the strong rejection of fully allogeneic islets by both perforin-deficient and wild-type animals. This was the case even if the grafts were derived from *lpr* donors, i.e., in the absence of Fas-mediated cytolysis,

and rejection proceeded in the absence of demonstrable *in vitro* cytolysis against the immunizing strain (Ahmed *et al.,* 1997). This was notwithstanding the clear reliance of graft rejection on $CD8^+$ T cells; for example, mice deficient in class I (Desai *et al.,* 1993) or treated with anti-CD8 antibodies (Walsh *et al.,* 1996) have prolonged graft survival. By contrast, perforin was critical for the development of autoimmune diabetes mellitus in NOD mice. Interestingly, diabetes mellitus was abrogated by perforin deficiency in a transgenic model of the disease (Kagi *et al.,* 1996). These mice developed typical insulitis with a strong inflammatory mononuclear infiltrate; however, islet cell destruction was markedly delayed. In studies of skin graft rejection (Selvaggi *et al.,* 1996), neither perforin nor Fas was critical for rejection, and it was surmised that alternative mechanisms assume importance in the effector phase of rejection when these cell-cytotoxic pathways are abrogated. By contrast, both perforin and Fas are likely to contribute to the chronic rejection of xenogeneic tumor grafts in mice (Smyth *et al.,* 1996b).

Braun and his colleagues (1996) studied the effects of both perforin and FasL deficiency on lethal graft versus host disease (GVHD) and concluded that both mechanisms were important, as delayed death occurred when either pathway was inoperative. These mice were also notable in that although lacking both conventional killing pathways, they were able to kill fibroblast cell lines through TNFα. In another study, immune suppression of the B cell compartment in allogeneic GVHD was dependent on Fas-mediated cytolysis by the engrafted T cells, but perforin deficiency was of no consequence. FasL on engrafted T cells was also responsible for eliminating radiation-resistant stem cells, and hematopoietic reconstitution with *gld* bone marrow therefore resulted in chimerism. FasL deficiency also affected the array of organs affected by GVHD in that FasL deficiency of the graft resulted in an absence of skin and hepatic lymphocyte infiltration and a reduction in the severity of gastrointestinal disease (Baker *et al.,* 1997; Spielman *et al.,* 1997). Nevertheless, Fas-ligand-deficient bone marrow transplantation did not prolong the survival of the recipient mice in this study. This was in contrast to bone marrow transplantation performed with perforin-deficient marrow, which resulted in a marked delay in GVHD (Spielman *et al.,* 1997).

The combined absence of perforin and FasL (i.e., in perforin-deficient *gld* mice) results in infertility in female mice and early death in both sexes, even under virus-free conditions (Spielman *et al.,* 1997). Many organs, particularly the exocrine pancreas, the uterus, and ovaries, are infiltrated with lymphocytes and then replaced with fibrotic tissues. Infiltrating cells were $CD3^+Thy1^+CD11b^+$ or $CD3^-Thy1^+CD11b^+$, suggesting expansion of the $CD11b^+$ T and macrophage populations. On the basis of these results, the authors postulated that perforin may be responsible for eliminating

some antigen presenting cells and that this is seen most obviously in the additional absence of FasL.

IV. Concluding Remarks

Much work over the past two decades has made it apparent that CL play an indispensible role in the immunopathology of a wide variety of conditions including tumor and allograft rejection, GVHD, and particularly in the response to intracellular pathogens. We have seen the discovery of two independently controlled cytolytic mechanisms which at first glance appeared to have little in common at the molecular level. And yet, recent advances in the broader field of apoptosis research have made it clear that the granzyme/perforin and FasL pathways overlap considerably at the biochemical and functional levels. Elucidation of two molecular pathways leading to cytolysis and parallel studies on the plethora of natural and synthetic compounds that can interfere with their execution has brought within our grasp the prospect of novel therapeutic agents that might modulate (either positively or negatively) the immune response in a variety of immunopathological situations. The remaining challenges for the future will be to fathom how the immune system chooses between the two pathways in a given pathological situation and to devise means of selectively and appropriately affecting the function of each.

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Drought-Induced Responses in Plant Cells

Zohreh Tabaeizadeh
Department of Biological Sciences, University of Quebec in Montreal, Montreal, Quebec H3C-3P8, Canada

Plants subjected to water stress undergo numerous physiological and metabolic changes. A general decrease in photosynthetic rate is among the most common responses. This is due to a programmed process involving the closure of stomata and reduction in the activity of photosynthetic enzymes. The plant hormone abscisic acid plays an important role in this process. Accumulation of compatible solutes, during water stress, is thought to be an adaptive response which has been developed by some plant species. Engineering the genes involved in the synthesis of these compounds, into nonaccumulating plants, has demonstrated promising results for genetic improvement of drought tolerance. Drought stress induces alteration of gene expression. A large number of genes which are upregulated by water stress have been isolated and characterized. Proteins encoded by some of these genes share several characteristics. The biochemical role of most of these gene products is unknown, but potential adaptive functions have been suggested. Abscisic acid is involved in the regulation of some of these genes.

I. Introduction

A plant's normal growth and function depend on a relatively high intracellular water content. As a consequence, plants subjected to drought stress experience a diverse set of physiological, metabolic, and developmental changes. Some of these changes can be the manifestation of damages which occur due to stress, while others can be potentially adaptive responses. In either case, these responses can vary depending on the severity of drought stress, the stage of development of the plant, and species. In this article we will mainly discuss plant cell responses which are induced by drought. However, other environmental stresses such as salinity and freezing temper-

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ature are also dehydration related and can evoke the same type of responses in plant cells as drought stress. For example, plant cells exposed to freezing temperature are dehydrated due to the presence of ice in extracellular spaces. Plants growing under saline conditions are water stressed due to the low water potential of the rooting environment. Therefore, whenever appropriate, besides drought stress, examples of cell responses to other dehydration-related conditions will also be highlighted.

In the studies which will be cited throughout this article, drought has been imposed by using different approaches. In some cases water has been withheld from plants for a defined period of time. In other cases detached leaves dehydrated on the laboratory bench (a condition which is far from the naturally occurring drought) have been used. Suspended cells grown in a medium containing high concentrations of mannitol, polyethylene glycol, or NaCl have also been used. All of these conditions lead to changes in different physiological and metabolic pathways which in most cases are controlled at the level of gene expression. These responses will be discussed in the first part of the chapter. The second part is devoted to drought-induced genes whose products do not have a known function.

II. Plant Cell Water Status

In order to study the plant–water relationship cell water content should be quantified. This has been a debatable subject since almost the second quarter of the nineteenth century. The measurement of water content through calculation of the percentage of fresh and dry weight can be considered the oldest approach for quantification of plant water status. Gradually, other approaches were developed, among which the measurement of cell water potential (Ψ_w) is the most often used. Plant cell water potential, which can be considered the free energy status of water in a plant cell, has been defined by the following equation (Kramer, 1983 and the references therein): $\Psi_w = \Psi_s + \Psi_p + \Psi_m + \Psi_g$, where Ψ_s represents osmotic potential, Ψ_p is the turger pressure, Ψ_m is matric potential (refers to the bound water), and Ψ_g represents the effect of gravity. The details on each of these components have been described in the past in books and reviews regarding plant–water relationships (Kramer, 1983) and, therefore, are not going to be discussed in this article. It is, however, important to note that in most cases the two last fractions (Ψ_m and Ψ_g) are not included in the equation; Ψ_m is considered to be negligible and Ψ_g is only important when the water potentials of leaves located at different heights in a tree are compared (Kramer, 1983).

In most of the studies regarding drought stress, plant water status has been monitored by measuring leaf water potential; however, it should be mentioned that different factors can affect the results. For example, the age of the leaves, their location on the stem, and the time of the day when the measurement is being carried out can make a difference. Due to these inaccuracies, the use of the appropriate descriptor of plant cell water status is still an issue of ongoing debate (Schulte, 1992).

Throughout this article, the degree of water stress is expressed in different ways such as leaf water potential, water content (percentage of dry or fresh weight), the number of days water has been withheld from the plant, etc. Also, the terms severe or moderate stress are sometimes used. The readers are invited to consult the respective references for more detail about the exact condition of drought stress.

III. Physiological and Biochemical Responses in Plant Cells during Drought Stress

A. Water-Stress-Induced Changes in Photosynthetic Activity

It is well documented that during drought stress photosynthetic activity is reduced. This reduction is due to several coordinated events such as the closure of stomata and a reduction in the activity of photosynthetic enzymes which are summarized below. The crassulacean acid metabolism, as a mechanism of adaptation to water stress, will also be discussed in this section.

1. Closure of Stomata

Stomatal closure during drought stress is thought to be triggered by abscisic acid (Zeevaart and Creelman, 1988). Indeed, most of the available ABA-deficient mutants demonstrate a "wilty" phenotype very early in the course of water stress. This has been suggested to be due to a defect in stomatal regulation (Neill and Horgan, 1985; Parry *et al.*, 1991). However, according to Hartung and Slovic (1991), the total ABA content per unit leaf area does not increase before the closure of stomata. These authors have suggested that there is a redistribution of ABA between different compartments. The cause of ABA redistribution is water-stress-induced compartmental pH shifts (Berkovitz *et al.*, 1983; Berkovitz and Gibbs, 1983; Hartung *et al.*, 1988; Hartung and Radin, 1989), which itself is due to the inhibition of proton motive forces such as ATPase at the plasmalemma (Hartung *et al.*, 1988). Indeed, a decrease of net proton fluxes of about 8.6 $nmol \cdot s^{-1} \cdot m^{-2}$

has been reported (Slovik *et al.*, 1992; Slovic and Hartung, 1992b). Slovik and Hartung (1992a,b) proposed a method for quantifying "stress" by the magnitude of compartmental pH shift. Some of their experimental data, which are presented in Table I, demonstrate compartmental pH shifts under stress. In their model the full pH shift indicates extreme stress and no pH deviation indicates unstressed conditions.

It has been reported that the closure of stomata starts as early as 2–5 min after the onset of drought stress (Hartung and Slovik, 1991). Several studies have been carried out in order to verify if the redistribution of ABA is significant enough to explain the stress-induced stomatal closure. Cornish and Zeevaart (1985) reported a 12-fold ABA increase in water-stressed *Xanthium strumarium* leaves after a loss of 12% of fresh weight. Hartung *et al.* (1988) observed increases up to 18-fold in the ABA concentration in the mesophyll apoplast of drought-stressed cotton leaves in the field. Slovik and Hartung (1992b) studied the kinetics of ABA redistribution among different leaf tissues and their compartments with regard to stomatal regulation during drought stress. They found up to 16.1-fold accumulation of ABA in guard cell walls after 6 h of drought stress (Fig. 1). This is much higher compared to that in the guard cell cytosol where only up to 3.4-fold ABA accumulation was observed. The authors concluded that the binding site of the guard cell ABA receptor faces the apoplasm. They also reported that a 2- to 3-fold ABA accumulation in guard cell walls is sufficient to

TABLE I

Compartmental pH Values and the Most Extreme pH Shifts Induced by Drought Stress (from Hartung and Slovik, 1991)

Compartment	Unstressed (pH)	Stress (pH)	Shift (ΔpH)
Apoplast			
Mesophyll	6.50	7.30	+0.8
Epidermis	6.00	7.50	+1.5
Guard cell	6.30	6.70	+0.4
Cytosol			
Mesophyll	7.30	7.15	−0.15
Epidermis	7.30[a]	7.15[a]	−0.15
Guard cell	8.16	7.97	−0.19
Phloem sap	7.60	7.45[a]	−0.15
Stroma			
Mesophyll	7.90	7.50	−0.4
Guard cell	8.20[a]	7.80[a]	−0.4

[a] Indicates estimated values.

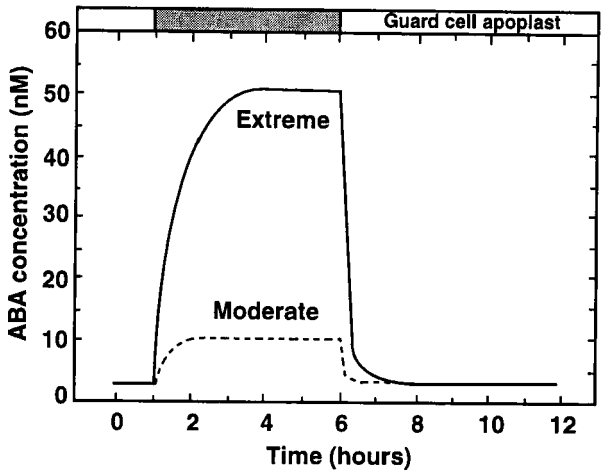

FIG. 1 The kinetics of the ABA concentration in guard cell walls of a model leaf for two magnitudes of stress; $S_r = 1.0$ (extreme) and $S_r = 0.33$ (moderate). From Hour 0 to Hour 1 there is no stress. At Hour 1.0, stress starts (cf. cross-hatches at the top) with shifting of pH values in all leaf compartments (Table I). The final pH value, which is achieved within 12 min, is defined by the magnitude of stress S_r. At Hour 6 stress ends, and all pH values return within 12 min to the pH values of unstressed leaf components. From Hartung and Slovik (1991).

induce closure of stomata. Considering these results, one could conclude that the accumulation of ABA in guard cells is extensive and fast enough to induce closure of stomata during drought stress.

2. Effect of Water Stress on Photochemical Reactions

In intact leaves exposed to water stress, a substantial decrease of O_2 evolution has been observed (Harvaux *et al.*, 1986), suggesting that water stress directly affects the photochemical reactions of photosynthesis. During the past few years more studies have been carried out on the effect of drought on photosystem II (PSII). This can be attributed to development of techniques for successful isolation of the PSII complex with the retaining ability of evolving O_2 and to the elucidation of the polypeptide composition of this photosystem (Mattoo *et al.*, 1989). He *et al.* (1995) investigated the effects of water stress on the metabolism of PSII polypeptides and the relation to PSII photochemical function. In part of their study using osmotically stressed wheat seedings they measured O_2 evolution and DCIP (2,6-dichlorophenol endophenol) photoreduction as indicators of PSII function. A mild water stress (reduction of 8.8% of relative water content and 0.08% of the leaf water potential, relative to control) caused only a slight change

of O_2 evolution and almost no change in DCIP photoreduction. Since a significant decrease in O_2 evolution was observed only when the osmotic stress became more severe, the authors suggested that the PSII is, to some extent, tolerant to water stress. The same authors reported a marked decline in the steady-state level of PSII high-molecular-weight (larger than 23 kDa) proteins, including the reaction center proteins D1 and D2. Comparing differing studies (Harvaux *et al.*, 1986; Harvaux, 1992; He *et al.*, 1995), one could conclude that PSII activity is not affected by mild water stress; however, it is inhibited by severe stress. As for the inhibition site, Harvaux *et al.* (1986) suggested that water stress primarily damages electron donation to PSII. This conclusion was based on the fact that the inhibition of PSII activity by drought could be reversed to a great extent by exogenous electron donors to PSII such as hydrazine and hydroxylamide. He *et al.* (1995), however, did not reach to the same conclusion. According to their results, the deterioration of PSII reaction center proteins can be accounted for as the main limiting factor for PSII function during drought stress.

3. Reduction of CO_2 Fixation

It has been shown that the activity of enzymes involved in CO_2 reduction is reduced during drought stress. This can be attributed, to some extent, to the reduction of synthesis of the corresponding mRNAs. A link with the increased level of ABA has again been demonstrated. Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), one of the key photosynthetic enzymes, has been the subject of several studies with regard to drought stress. Bartholomew *et al.* (1991) examined the effects of an increased level of leaf endogenous ABA on the expression of rbcS (the nuclear gene-s coding for the small subunit of Rubisco). In order to carry out this study they used water stress on a tomato ABA-deficient mutant and its wild-type progenitor. In regularly watered plants the ABA level in the mutant genotype was 15% that of the wild type. Upon water stress the ABA level increased 10-fold in the wild genotype; only a slight increase was detected in mutant plants. In the drought-stressed wild genotype, rbcS mRNA levels were only 12% of control, whereas only a slight decrease was observed in the mutants' rbcS mRNA level. When the same blots were probed with rbcL (the chloroplast gene coding for the large subunit of rubisco), no difference was observed between the irrigated and water-stressed plants of either line. The authors also examined the drought-stressed plants which have been rewatered. They concluded that during recovery from stress, ABA returns to the control level; however, the expression of rbcS is induced to levels higher than those in nonstressed plants. By conducting nuclear run-on experiments, it was demonstrated that the negative effect of water stress on the rbcS mRNA level is primarily at the

transcriptional level. The effect of water stress has also been examined on the expression of cab genes (coding for chl a/b-binding proteins). The cab steady-state mRNA levels droped to 70% in drought-stressed tomato plants compared to that of the regularly watered plants (Bartholomew *et al.*, 1991).

The activity of fructose 1,6-biphosphatase has also been shown to be inhibited by water stress (Berkovitz and Gibbs, 1983). This inhibition was suggested to be mediated by stromal acidification, which in turn is facilitated by osmotically induced chloroplast shrinkage.

4. Crassulacean Acid Metabolism (CAM) and CAM Idling

CAM is a photosynthetic pathway which can be considered a biochemical and physiological adaptation to those environmental stresses which limit water availability (Ting, 1985; Osmond and Holtum, 1981). In CAM plants stomata open at night (during which the transpiration rate is low), allowing nocturnal CO_2 fixation. This process during which phosphoenol pyruvate transforms to C4 acids (mainly malate) is catalyzed by phosphoenol pyruvate carboxylase (PEPCase). The C4 acids are stored in the vacuole. During the following day, while stomata are closed, C4 acids are decarboxylized. This decarboxylation provides CO_2 for refixation by ribulose biphosphate carboxylase (through the Calvin cycle), and phosphoenol pyruvate for starch biosynthesis (through gluconeogenesis). The CAM pathway has significant advantages for plants. The loss of water is reduced since the gas exchange is carried out only during nighttime when the temperature is lower. Moreover, the activity of CAM-specific enzymes results in an increased CO_2 concentration (Ting, 1985).

Mesembryanthemum crystallinum (common ice plant) is a facultative halophyte in which the ability to perform CAM is inducible. This species switches from C_3 to CAM during a later stage of its development. This transition is accelerated by environmental stresses such as drought and salt. Several studies have been carried out in order to understand the mechanism of the transition from C_3 to CAM. PEPCase has been the subject of few of these studies. The level of this enzyme increases from approximately 1% of the total soluble protein in young plants to 10% of the soluble protein in mature salt-stressed leaves (Winter *et al.*, 1982b). A correlation between the increase in PEPCase activity and the increase in an approximately 100-kDa polypeptide has been demonstrated (Foster *et al.*, 1982; Winter *et al.*, 1982a). Based on the molecular weight and intracellular location, the 100-kDa polypeptide was suggested to be PEPCase (O'Leary, 1982). The induction of PEPCase by salt stress in *M. crystallinum* has been reported to be influenced by the age of the plant (Ostrem *et al.*, 1987). This induction was shown to be through an increase in the steady-state level of translatable mRNA for the enzyme.

The genes encoding CAM-specific PEPCase (*Ppc1*) and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (*Gap1*) have been studied. Both genes were shown to be transcriptionally upregulated in *M. crystallinum* during salt stress (Cushman *et al.,* 1989; Ostrem *et al.,* 1990; Vernon *et al.,* 1993). However, CAM induction in ice plant seems to be a complex process. High salinity can induce CAM only in plants which are approximately more than 5 weeks old. Indeed, in plants less than 4 weeks old, there is a very low level of *Ppc1* induction. Therefore, CAM induction in young ice plant is incomplete (Cushman *et al.,* 1990; Vernon *et al.,* 1993; Bohnert *et al.,* 1994). The expression of *Ppc1* is also controled by photoperiod (Cheng and Edwards, 1989), light intensity (McElwain *et al.,* 1992), and plant hormones such as cytokinin and abscisic acid (Chu *et al.,* 1990; Dai *et al.,* 1994; Thomas *et al.,* 1992; Vernon *et al.,* 1993).

A gene (*Pgm1*) encoding cofactor-independent phosphoglyceromutase was isolated from ice plant (Forsthoefel *et al.,* 1995). This enzyme catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis and gluconeogenesis. *Pgm1* expression increases in leaves of plants exposed to either saline or drought conditions. *Pgm1* mRNA accumulation in leaves has also been observed in response to treatment with either abscisic acid or cytokinin. The water-stress-induced increase in *Pgm1* steady-state mRNA in leaves was shown to accompany the switch from C_3 to CAM. The induction of *Pgm1* by salt and drought stress has been proposed to be the demonstration of the fact that the adaptation of ice plant to water stress involves adjustments in carbon metabolism pathways (Forsthoefel *et al.,* 1995).

Another interesting photosynthetic adaptation to water stress is a metabolic state termed CAM idling. Many CAM plants when severely water stressed switch from CAM to CAM idling, in which stomata are closed day and night; nevertheless, there is a continued low diurnal fluctuation of organic acids (Rayder and Ting, 1983a). The organic acids are apparently synthesized by refixation of respiratory CO_2 (Patel and Ting, 1987). *Xerosicyos danguy* is an example of a CAM plant which switches to CAM idling upon water stress (Rayder and Ting, 1983b,c). Water stress was shown to cause an increase in phosphoenol pyruvate carboxylase mRNA, enzyme activity, and PEPCase protein in this plant (Bastide *et al.,* 1993). This is very interesting considering the fact that the plant has already been in CAM. This increase was suggested to be the consequence of the increase in ABA concentration in response to water stress (Bastide *et al.,* 1993). This hypothesis is supported by the fact that the treatment of ice plant with ABA causes the shift from C_3 to CAM accompanied by expression of PEPCase (Chu *et al.,* 1990).

B. Accumulation of "Osmolytes"

Synthesis of the molecules termed osmoprotectants, osmolytes, or compatible solutes can be accounted as one of the mechanisms that plants have evolved for adaptation to water deficit. These molecules which act as osmotic balancing agents are accumulated in plant cells in response to salt or drought stress and are subsequently degraded after stress relief. A dominant class of osmoprotectants includes amino acids (such as proline), quaternary ammonium, and tertiary sulfonium compounds (Rhodes and Hanson, 1993).

Accumulation of free amino acids, particularly proline, has been suggested to play a role in plant cell adaptation to osmotic stress (Yancey *et al.,* 1982; Schobert, 1977; Delauney and Verma, 1993). Proline has been shown to act as a solute that protects proteins during dehydration (Paleg *et al.,* 1984). In bacteria proline is synthesized from glutamate. Higher plants synthesize proline via two different pathways (Bryan, 1990). The first pathway is from glutamate, where it is converted to Δ^1-pyrroline-5-carboxylate (P5C) by Δ^1-proline-5-carboxylate synthetase. P5C is then reduced to proline by Δ^1-pyrroline-5-carboxylate reductase. The second pathway for proline biosynthesis in higher plants is from ornithine, where it is transformed to Δ^1-pyrroline-5-carboxylate through δ-transamination. P5C is subsequently reduced to proline.

Studies have been particularly carried out using *in vitro* cultured cells. In the cells adapted to 30% of polyethylene glycol (PEG), proline represents over 50% of the total free amino acid pool (Handa *et al.,* 1983). Alanine and γ-aminobutyrate accumulate also in the water-stress-adapted *in vitro* cultured cells (Handa *et al.,* 1983). The same amino acids also accumulate in the whole plant in response to water deficit (Hanson and Hitz, 1982; Stewart and Larher, 1980). The *in vivo* rates of synthesis, utilization, and compartmentation of free amino acid pools were determined in control and water-stress (25% PEG)-adapted *in vitro* cultured cells of *Lycopersicon esculentum* (Table II; Rhodes *et al.,* 1986). It was concluded that proline synthesis via the glutamate pathway is increased 10-fold in response to adaptation to water stress. About 8% of the newly synthesized proline is catabolized in unadapted (control) cells, but only 4.7% in adapted cells. It was hypothesized that the water-stress-adapted cells develop mechanisms which limit the oxidation of proline. The same type of conclusion had been drawn in other studies as well (Huang and Cavalieri, 1979; Stewart *et al.,* 1977). It was also concluded that the main pathway for proline biosynthesis in water-stress-adapted cells is through glutamate and not ornithine. Adaptation to water stress leads to increased nitrogen flux from glutamate into alanine and γ-aminobutyrate. This can suggest increased pyruvate availability and increased rates of glutamate decarboxylation (Rhodes *et al.,* 1986).

TABLE II

Amino Acid Pool Sizes (μmol/g Fresh Weight) of Tomato Cell Suspension Cultures Adapted to Control Medium or to the Medium Containing 25% Polyethylene Glycol

Amino acid[a]	Control[b]	PEG[b]
Alanine	2.020	6.26
γ-Aminobutyrate	1.23	13.00
Arginine	0.06	0.256
Glutamate	0.844	1.215
Glutamine	6.77	2.711
Homoserine	0.048	0.465
Isoleucine	0.080	0.680
Ornithine	0.035	0.063
Proline	0.120	31.20
Serine	0.780	3.63
Threonine	0.083	0.71
Tyrosine	0.062	0.349
Valine	0.65	15.315

Note. Data from Rhodes *et al.*, 1986.

[a] Only those amino acids for which an important difference in concentration, between control and PEG-adapted cells, has been found are included.

[b] Each concentration is the average of 8 values resulting from measurements conducted at 8 different times, 0.03–48.0 h in culture.

In water-stress-adapted cells both alanine and γ-aminobutyrate are synthesized at higher rates compared to the amount which is normally required. This indicates that these amino acids are rapidly turned over (Rhodes *et al.*, 1986).

The quaterernary ammonium and tertiary sulfonium compounds (QACs and TSCs) which accumulate in higher plants include glycine betaine, alanine betaine, proline betaine, hydroxyproline betaine, dimethylsulfoniopropionate, and choline-*O*-sulfate. Numerous studies with regard to the mechanism of cellular adaptation to osmotic stress have been concentrated on the synthesis and the role of these osmolytes. Initially, bacteria played an important role in these studies. Indeed, it has been reported that several of the molecules that accumulate during osmotic stress in plants can also act in osmotically stressed bacteria as osmoprotectants (Le Rudulier *et al.*, 1984, and references therein). For example, it has been shown that the bacterium *Klebsiella pneumoniae* can grow in the presence of a normally inhibitory level of NaCl (0.8 *M*) when glycine betaine was added to their medium (Le Rudulier and Valentine, 1982). Even at a concentration as low as 10^{-5} *M* glycine betaine could stimulate growth. Among 150 different

metabolites tested on *Escherichia coli,* only the betaine series have proven potent in stimulating growth under inhibitory levels of osmotic stress. The most active ones were found to be glycine betaine, choline, and proline betaine. Free proline has also been found to be active, but under lower levels of osmotic stress (Le Rudulier *et al.,* 1984).

As to how osmolytes work, their accumulation has been shown to be correlated with tolerance to water deficit conditions caused by drought or salinity. These molecules function in two different ways. First, they act as nontoxic osmolytes, allowing cells to balance the osmotic strength of its cytoplasm with that of the surrounding environment. Second, they influence the stability of enzymes and membranes during osmotic stress (Wyn Jones, 1984; Warr *et al.,* 1988). *In vitro* incubation of osmolytes with plants' protein extracts has been shown to reduce the negative effects of electrolytes on enzyme activity (Pollard and Wyn Jones, 1979; Manetas *et al.,* 1986; Krall *et al.,* 1989).

Quarternary ammonium and tertiary sulfonium compounds are accumulated to considerable levels (5 μmol g^{-1} dry weight) by certain higher plant genera during water deficit (Rhodes and Hanson, 1993). Glycine betaine is the most widespread of these compounds. Its synthesis has been best characterized in members of the Chenopodiaceae (spinach and sugar beet) and the Gramineae (barley and maize). In these plants glycine betaine is synthesized via a two-step oxidation of choline: choline → betaine aldehyde → glycine betaine (Hanson and Hitz, 1982; Rhodes and Hanson, 1993). The first step is catalyzed by choline monooxygenase (CMO) (Brouquisse *et al.,* 1989); the second step, by betaine aldehyde dehydrogenase (BADH) (Weigel *et al.,* 1986; Arakawa *et al.,* 1992). Both CMO and BADH have been shown to be soluble chloroplast enzymes in spinach and are induced by water stress caused by salinity or drought (Weigel *et al.,* 1986; Brouquisse *et al.,* 1989). Complementary DNA clones encoding BADH from spinach (Weretilnyk and Hanson, 1990), sugar beet (McCue and Hanson, 1992), and barley (Ishitani *et al.,* 1995) have been isolated. As for CMO, the enzyme from spinach was purified and partially characterized (Burnet *et al.,* 1995).

Accumulation of QACs and TSCs does not occur in all plant genera (Rhodes and Hanson, 1993). Moreover, different types have been found in accumulating species. Comparative biochemical and immunological studies have led to the conclusion that glycine betaine biosynthesis has appeared earlier in angiosperm evolution compared to that in β-alanine betaine, choline-*O*-sulfate, proline betaine, and hydroxyproline betaine (Weretilnyk *et al.,* 1989; Ishitani *et al.,* 1993; Rhodes and Hanson, 1993). The fact that in those species who have been studied, nevertheless, a small amount of glycine betaine and even sometimes low levels of BADH protein have been traced in nonaccumulating plants has led to a hypothesis suggesting that

all plants may have genes for betaine synthesis (Weretilnyk *et al.,* 1989). Another interesting point is that both choline and glycine betaine have been found to stimulate growth of certain plant pathogenic fungi such as *Fusarium graminearum,* which causes fusariose in some cereals (Strange *et al.,* 1974; Pearce *et al.,* 1976). This disadvantage of the glycine betaine trait has been suggested to have contributed to the evolution of alternative compatible solutes (Rhodes and Hanson, 1993) in some species so that they can avoid fungal attack.

A detailed study on a highly stress tolerant family, Plumbaginaceae, showed that different members of this family have evolved four different QACs, which supplement or replace glycine betaine (Hanson *et al.,* 1994). These are choline-*O*-sulfate, β-alanine betaine, proline betaine, and hydroxyproline betaine. The results of bacterial bioassays demonstrated that these QACs are not more efficient, as osmoprotectants, compared to glycine betaine. Interestingly, different environmental stresses favor the accumulation of different QACs in members of this family. For example, proline-derived betaines are accumulated by species growing in dry environments, β-alanine betaine accumulates in species growing under saline conditions, and choline-*O*-sulfate accumulates in species growing in soil containing sulfate. It was therefore suggested that different QACs might have selective advantages for plants growing in particular abiotic stress (Hanson *et al.,* 1994). If this is proven it could be possible to engineer plant metabolic pathways, so that they can tolerate individual types of environmental stresses.

Another group of osmoprotectants are polyols (Yancey *et al.,* 1982). Polyols are either straight chain, such as mannitol and sorbitol, or cyclic, such as *myo*-inosytol and its methylated derivatives (Bieleski, 1982; Loewus and Dickinson, 1982). Accumulation of mannitol and sorbitol has been reported to be correlated with drought tolerance (Bieleski, 1982). Mannitol, especially, has been found in a wide range of crop plants (Stopp *et al.,* 1996). *In vitro,* it can act as hydroxyl radical scavenger (Franzini *et al.,* 1994). Both mannitol and sorbitol can also be effective in osmotic adjustment by helping retention of water in the cytoplasm.

C. Carbohydrate Metabolism

The studies regarding changes in carbohydrate metabolism in plants exposed to drought stress were primarily inspired by some reports on animal systems. Early studies had suggested that under dry conditions the hydroxyl group of polyhydroxy compounds can form a hydrogen bond to polar heads of membrane phospholipid, the hydrophobic interactions which are important for membrane stability (Crowe *et al.,* 1984a). Strong experimental

evidence for this hypothesis was provided later by Strauss and Hauser (1986) who added Eu^{3+} ions to preparations of phosphatidylcholine vesicles and sucrose. Eu^{3+} normally forms an ionic bond with the phosphate of phospholipids. In the experiment the stabilization of the vesicles by sucrose during freeze drying was significantly reduced when the Eu^{3+} ions were added. It was suggested that under this condition a competition establishes between sucrose and Eu^{3+} for binding to the phosphate head group of the phospholipids (Strauss and Hauser, 1986).

Using ca-transporting microsomes from muscle as a model membrane it was demonstrated that trehalose, a nonreducing disaccharide of glucose, can preserve the structural and functional integrity of membranes under low-water conditions (Crowe *et al.,* 1984b). Other sugar and sugar alcohols such as lactose, maltose, cellobiose, sucrose, and glucose were also tested in this study; they were less effective. Studies on yeast, fungi, and bacteria have also correlated accumulation of trehalose and desiccation tolerance. Overall, in all of these systems, trehalose has been found to be the most effective sugar with regard to the minimum concentration required to act as an osmoprotectant (Crowe *et al.,* 1992). The occurrence of trehalose in plants is rare. Some other sugars and particularly sucrose seem to be a replacement for trehalose in angiosperm seeds (Leprince *et al.,* 1993). These seeds can withstand desiccation during a specific period of their development. The desiccation tolerance is lost once the radicle starts to emerge (Senaratna and McKersie, 1983). In soybean, pea, and corn seeds, some soluble sugars such as sucrose, raffinose, and stachyose are present during the desiccation tolerance stage. In general, the loss of desiccation tolerance is accompanied by a sharp decrease in the level of these polysaccharides. A significant increase in the level of glucose is also detected at the same time (Koster and Leopold, 1988). It should be mentioned that the level of stachyose and raffinose decrease before that of sucrose. A collaborative role of these soluble sugars against water stress in seeds has been suggested. Sucrose by itself, as mentioned earlier, can replace water by an interaction with a hydrophilic group of membrane phosolipids. However, during slow drying sucrose may either crystallize or form an amorphus glass. If it crystallizes, the hydroxyl groups will become unavailable. In contrast, when it forms glass, the hydroxyl groups stay free and can bind to the hydroxyl part of membrane phospholipid through a hydrogen bond (Koster and Leopold, 1988). It has been suggested that in the presence of oligosaccharides such as raffinose and stachyose, sucrose forms glass instead of being crystallized (Caffrey *et al.,* 1988; Koster, 1991). This hypothesis is supported by the fact that, raffinose, for example, can prevent the crystallization of sucrose (Caffrey *et al.,* 1988).

Accumulation of sucrose in *Craterostigma plantagineum* during dehydration stress adds more evidence for the possible role of this sugar in drought

tolerance. This resurrection plant is extremely tolerant to drought. It can loose 95% of its water and yet fully recover within a few hours after rehydration. In fully hydrated leaves of *C. plantagineum,* 89% of the water-soluble carbohydrates is 2-octulose, an eight-carbon sugar. On the other hand, sucrose represents 90% of the total sugar content in the leaves of 5-day drought-stressed plants (Bianchi *et al.,* 1991). A high level of sucrose during water stress conditions is accompanied by marked increases in the quantity and activity of sucrose synthase and sucrose phosphate synthase (Elster, 1994, in Bernacchia *et al.,* 1995). Upon rehydration the level of 2-octulose increases, while the sucrose level drops (Bianchi *et al.,* 1991). Interestingly, the accumulation of sucrose–phosphate synthase activity, during water stress, has also been reported in spinach, which is not a drought-tolerant species (Quick *et al.,* 1989).

cDNA clones encoding three classes of transketolase have been isolated from *C. plantagineum* (Bernacchia *et al.,* 1995). Transketolases are key enzymes which have several functions in intermediary metabolism. They are involved in the degradation and synthesis of carbohydrates in the oxidative and reductive pentose phosphate cycles. In these cycles different sugar phosphate intermediates are synthesized which can be used in carbohydrate metabolism or nucleic acid biosynthesis (Racker, 1961). Among the transketolase cDNAs isolated from *C. plantagineum,* one class is constitutively expressed, at the transcriptional level. However, the expression of two other classes is associated with the rehydration process of drought-stressed plants. As mentioned earlier, the conversion of sugars is a phenomenon observed during dehydration–rehydration of *C. plantagineum* (Bianchi *et al.,* 1991). Therefore, it is possible that these two classes of transketolases play a role in this conversion, during the rehydration process.

D. Osmoprotectants and Genetic Engineering of Plants

Since the "osmoprotectants" are not accumulated in all plant species and their synthesis and accumulation are correlated to water-deficit tolerance, it has been proposed that engineering their synthesis pathway into a nonaccumulating species will increase tolerance to salt or drought stress. This has already been demonstrated for *Synechococcus sp.* PCC7942, a freshwater cyanobacterium which was transformed by *E. coli* genes for the synthesis of glycine betaine (Nomura *et al.,* 1995). Under conditions of salt stress the transformed cells accumulated glycine betaine to a concentration of 45 m*M,* and this resulted in stabilization of the photosynthetic activities of both photosystems I and II and general protective effects against salt stress.

As for higher plants, few examples can be cited (Table III). Polyol functions were tested by introducing the bacterial *mtlD* gene (encoding

TABLE III

Examples of Transformation Experiments Leading to Accumulation of One of the "Osmoprotectants" and an Apparent Improvement in Water-Stress Tolerance

Gene transferred[a]	Metabolite accumulated in transgenic tobacco plants	Reference
mtlD from *Escherichia coli* encoding mannitol-1-phosphate dehydrogenase	Mannitol	Tarczynski *et al.* (1992, 1993)
Δ^1-pyrroline-5-carboxylate synthetase from mothbean	Proline	Kishor *et al.* (1995)
SacB from *Bacillus subtilis* (encoding levanosucrase)	Fructane	Pilon-Smits *et al.* (1995)
Trehalose-6-phosphate synthase subunit of trehalose synthase from yeast	Trehalose	Holmström *et al.* (1996)

[a] In all cases the coding sequence of the gene has been linked to a constitutive promoter except for the trehalose synthase gene where the promoter of the Rubisco small subunit gene has been used.

mannitol-1-phosphate dehydrogenase) under the control of a constitutive promoter into tobacco. This species normally does not synthesis mannitol. Expression of *mtlD* and accumulation of mannitol were demonstrated in the transgenic plants (Tarczynski *et al.*, 1992). Moreover, when these plants were exposed to salt stress, they exhibited some degree of tolerance measured by plant height, plant fresh weight, and root growth (Tarczynski *et al.*, 1993).

The case for glycine betaine seems to be more complicated. This is due to the fact that, as mentioned earlier, its biosynthesis is carried out via a two-step pathway: choline → betaine aldehyde → glycine betaine. Betaine aldehyde dehydrogenase (the enzyme catalyzing the second reaction) cDNAs from sugar beet and spinach were transferred to tobacco, which lacks both enzymes necessary for the synthesis of glycine betaine (Rathinasabapathi *et al.*, 1994). The rationale for transferring a gene encoding BADH prior to the one encoding choline monooxygenase (the enzyme catalyzing the first reaction) is that betaine aldehyde is toxic to plants. The BADH level in transgenic plants was comparable to those in spinach and sugar beet. Two points are worth mentioning. First, despite the absence of a transit peptide, BADH was targeted to the chloroplast in leaves of transgenic tobacco plants. Second, transgenic tobacco plants absorbed and oxidized exogenous betaine aldehyde and accumulated glycine betaine to levels similar to those found in drought-stressed or salinized spinach and sugar beet. This demonstrates the fact that in transgenic tobacco betaine aldehyde

is transported across the plasmalemma and chloroplast envelope like that in spinach and sugar beet.

The gene encoding mothbean Δ^1-pyrroline-5-carboxyate synthetase was transferred to tobacco under the control of a constitutive promoter (Kishor *et al.,* 1995). Transgenic plants demonstrated high activity of the enzyme and produced a higher level of proline compared to control plants. It was suggested that Δ^1-pyrroline-5-carboxyate synthetase is the rate-limiting factor for proline biosynthesis. This hypothesis was based on the fact that overproduction of Δ^1-pyrroline-5-carboxyate reductase did not increase production of proline in transgenic plants (Szoke *et al.,* 1992). Under drought and salinity conditions, the roots of transgenic plants had more biomass and flower production compared to those in control plants.

A gene encoding the trehalose-6-phosphate synthase subunit (TPS1) of yeast trehalose synthase was transferred to tobacco. TPS1-positive plants accumulated 0.8–3.2 mg (per gram dry weight) trehalose, while control plants contained only 0.06 mg. Self progeny of one TPS1-positive line was demonstrated to tolerate dehydration when air dried for 7 h, in contrast to the control plants, which were completely wilted (Holmström *et al.,* 1996).

IV. Effects of Drought on Oxidative Stress and Gene Expression

A. Drought-Induced Oxidative Stress

Besides its primary effect on photosynthesis, the closure of the stomata during drought conditions generates oxidative stress. The decrease in CO_2 availability for photosynthesis leads to misdirection of electrons in the photosystems, which in turn results in the formation of reactive oxygen species, superoxide radicals (O_2). In the presence of metal ions such as iron, superoxide can react with hydrogen peroxide. This reaction, which is known as the Haber–Weiss reaction (Bowler *et al.,* 1992), results, in part, in the formation of hydrxyl radicals (OH^-), which can be considered one of the most harmful reactive species (Cadenas, 1989). Hydroxyl radicals can cause mutation, lipid peroxidation, and protein denaturation (Halliwell, 1987; Cadenas, 1989). Reactive oxygen species are not only produced during drought but also under many other stress conditions. Low temperature, high light intensity, and treatment with some herbicides such as paraquat can be cited as a few examples (Bowler *et al.,* 1992). It has been suggested that the mechanisms which reduce oxidative stress might have, indirectly, a role in increasing plant tolerance to these stresses (Bowler *et al.,* 1992). There is no known mechanism to eliminate the hydroxyl radicals, once they are formed. However, their formation can be prevented through the

following reaction: $2O_2^- + 2H \rightarrow O_2 + H_2O_2$. This reaction, which is catalyzed by the enzyme superoxide dismutase, leads therefore to a reduction in the availability of superoxide radicals. Superoxide dismutase, therefore, plays a crucial role in protecting plants against oxidative stress. There are three types of superoxide dismutase, depending on their metal cofactor (Bannister *et al.,* 1987). These are FeSOD, MnSOD, and Cu/ZnSOD. In plants in general, MnSOD is localized in mitochondria, FeSOD in chloroplast, and Cu/ZnSOD in cytosol as well as in chloroplast (Bannister *et al.,* 1987).

One of the reactions by which a cell can dispose of hydrogen peroxide is catalyzed by the enzyme catalase which in plants is found predominantly in peroxisomes. Through this reaction hydrogen peroxide is transformed to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$).

Another enzyme which is important in reducing the level of hydrogen peroxide is peroxidase. A chloroplast localized ascorbate peroxidase along with dehydroascorbate reductase and glutathione reductase are especially known to remove H_2O_2 by a mechanism called the Halliwell–Asada pathway (Foyer and Halliwell, 1976; Asada and Takahashi, 1987; Halliwell, 1987).

Some of the enzymes implicated in scavenging of superoxide radicals and of hydrogen peroxide have been studied in the context of drought stress. For example, in drought-stressed wheat and cotton plants, glutathione reductase activity increases (Gamble and Burke, 1984; Burke *et al.,* 1985). In tomato, drought stress leads to a strong induction of cytosolic Cu/ZnSOD (Bowler *et al.,* 1992). Using maize inbreds with different levels of drought tolerance, Malan *et al.* (1990) found a correlation between drought tolerance and activity of the enzymes Cu/ZnSOD and glutathione reductase. It is important to mention that the induction of enzymes involved in scavenging superoxide and hydrogen peroxide by drought depends on how severe the stress is. In maize plants exposed to moderate drought conditions no change in ascorbate peroxidase or glutathione reductase activity was observed in mesophyll cells. In bundle sheat cells a slight increase was observed in the activity of glutathione reductase, while ascorbate peroxidase remained unchanged (Brown *et al.,* 1995). It is possible that when drought stress is imposed gradually, as it was the case of this study, acclimation occurs; in that case, the level of H_2O_2 does not increase (Brown *et al.,* 1995). SOD activity has not been verified in this study; however, Sgherri *et al.* (1993) have suggested that in drought-acclimated sunflower plants, the level of superoxide probably does not increase. In the leaves of spinach plants severely drought stressed (loss of 50% fresh weight compared to control plants) the activity of ascorbate peroxidase and glutathione reductase increased significantly (Tanaka *et al.,* 1990).

Due to the major role of SOD in scavenging superoxide radicals and its induction during different environmental stresses, efforts have been directed toward overproduction of this enzyme in some plant species, through genetic engineering. Transgenic tomato and tobacco plants that overexpress a chloroplastic Cu/ZnSOD derived from petunia were produced. These plants, however, when exposed to some stress conditions which lead to oxidative stress, showed the same level of susceptibility as the control plants (Tepperman and Dunsmuir, 1990). As suggested by these authors, in order to protect plants against oxidative stress, besides SOD, the overproduction of at least an enzyme (such as catalase) involving in detoxification of H_2O_2 is necessary. The increased activity of SOD leads to an increase in the level of H_2O_2 which, by itself, is toxic to the cell.

Overproduction of MnSOD in tobacco chloroplast conferred protection against oxidative stress generated by treatment with the herbicide paraquat (Bowler *et al.,* 1991). The difference between the results of this study and those of the one cited before (Tepperman and Dunsmuir, 1990) was attributed to the different level of sensitivities of the two enzymes to H_2O_2 (Bowler *et al.,* 1992). Gupta *et al.* (1993) produced transgenic plants expressing a gene that encodes a chloroplast that localized Cu/ZnSOD from pea. Exposure to severe stress (caused by high light intensity and low temperature) reduced the rate of photosynthesis in both transgenic and nontransgenic tobacco plants, at almost a similar rate. However, when the stressed plants were exposed to normal conditions, the photosynthetic rate of transgenic plants was 90% of that before stress, while that in nontransgenic plants was only 36%. This demonstrates that severe oxidative damage which cannot be easily repaired had occurred in nontransgenic plants, while in transgenic plants, the oxidative damage was nonsignificant.

In the cited examples no evidence on the improvement of transgenic plants for drought tolerance has been presented. More recently, McKersie *et al.* (1996) reported water-deficit tolerance of transgenic alfalfa plants overproducing Mn-SOD. These transgenic plants when subjected to drought stress demonstrated less injury compared to that in control plants, as measured by chlorophyll fluorescence, electrolyte leakage, and regrowth from crowns. The transgenic plants also had a significantly higher yield than the control plants when tested in the field.

B. Drought-Induced Alteration of Gene Expression

Studies on various species have demonstrated that during drought, total protein synthesis is not reduced. However, drought stress causes an increased, and in some cases reduced, abundance of discrete protein and mRNA species. Therefore, isolation of the genes which are upregulated

by water stress has been used as one of the strategies for studying the plant response to drought. This approach has led to the identification of quite a number of genes. Some of these genes encode proteins which are known to be involved in specific metabolic pathways, outlined in the previous sections. On the other hand, a number of genes have been isolated which are highly induced by water stress; however, the encoded protein does not have any known function. Among the studies which have been undertaken with regard to drought-induced genes, some have used drought-tolerant species aiming to identify genes which can be implicated in drought tolerance. Some other studies have used seeds. This is due to the fact that seeds undergo extreme desiccation during the last phase of maturation, having a water content of only 5–10%. There are also numerous studies using species which are not necessarily drought tolerant. Nevertheless, these studies have led to identification of genes with interesting features and have increased our knowledge on general plant response to water stress. A list of the best characterized drought-induced genes (whose protein does not have a known function) which have been screened from cDNA libraries constructed from drought-treated tissues is presented in Table IV. The genes which have been isolated from seeds are not included in the table.

Interestingly, in spite of their origin, the proteins encoded by quite a number of these genes are similar in several ways such as hydrophylicity, boiling solubility, and responsiveness to ABA treatment and an absence of cysteine and trypthophan residues. Moreover, different conserved amino acid motifs exist in several of these genes. It has been hypothesized that the conserved sequences of these proteins may be critical to their function in the plants' response to dehydration. Each of the isolated genes apparently belongs to a small multigene family. In most cases, drought-induced changes of mRNA can be reversed by subsequent rehydration. Some of the best characterized of these genes will be reviewed in this section.

1. Upregulation of Genes Encoding Proteins with Unknown Function in Drought-Tolerant Species

Some plant species can withstand severe drought stress. *C. plantagineum* is one of the best examples. This resurrection species can survive long periods of desiccation (water losses higher than 90%) and resume complete metabolic activity a few hours after rehydration (Gaff, 1971; Bartels *et al.*, 1990).

Five cDNA clones representing the desiccation-related transcripts were isolated from this species. These cDNA clones are highly expressed in desiccated leaves and roots of *C. plantagineum,* while no expression (except for pcC27-04 where a basic level of expression is found in untreated roots) can be found in untreated leaves and untreated roots (Piatkowski *et al.,*

TABLE IV

Expression Characteristics of Some of the Drought-Induced Genes[a]

Species	Drought treatment[b]	cDNA clone	Induced in[c]	ABA[d]	Reference
Hordeum vulgare	DS	dhn1	Whole seedling	NR	Close *et al.* (1989)
		dhn2	Whole seedling		
		dhn3	Whole seedling		
		dhn4	Whole seedling		
Zea mays	DS	dhn1	Whole seedling	NR	Close *et al.* (1989)
Craterostigma plantagineum	ADL	pcC6-19	Leaf	+	Piatkowski *et al.* (1990)
		pcC3-06	Leaf	+	
		pcC27-45	Leaf	+	
		pcC13-62	Leaf	+	
		pcC27-04	Leaf	+	
Lycopersicon esculentum	ADL	pLE4	Leaf	+	Cohen and Bray (1990)
		pLE16			
		pLE25			
Hordeum vulgare	W	Paf93	Whole seedling	−	Grossi *et al.* (1992)
Arabidopsis thaliana	ADP	RD19	Whole plant	−	Yamaguchi-Shinozaki *et al.* (1992)
		RD22	Whole plant	+	
		RD29	Whole plant	M	
Pisum sativum	DS	pPsB12	Whole seedling	+	Robertson and Chandler (1992)

Lycopersicon chilense	W	pLC30-15	Leaf, stem, and root	+	Chen and Tabaeizadeh (1992a), and Chen *et al.* (1993)
Lycopersicon chilense	W	pcht28	Leaf and stem	+	Chen *et al.* (1994)
Triticum durum	DS	pTd27e pTd25a	Root and shoot	+	Labhilili *et al.* (1995)
Arabidopsis thaliana	W	AtDi8	Leaf and root	+	Gosti *et al.* (1995)
		AtDi19	Leaf and root	−	
		AtDi21	Leaf and root	+	
Solanum chacoense	ADP	DS2	Leaf, stem	M	Silhavy *et al.* (1995)
Helianthus annuus	W	sdi-1	Leaf	−	Ouvrard *et al.* (1996)
		sdi-5	Leaf	M	
		sdi-6	Leaf	−	
		sdi-8	Leaf	+	
		sdi-9	Leaf	+	
		sdi-10	Leaf	+	
Lycopersicon pennellii	W	His1	Leaf	+	Wei and O'Connell (1996)

[a] Only the genes which have been screened from cDNA libraries constructed from drought-treated plants, seedlings, or leaves are included.

[b] Treatments which have been used for the construction of cDNA library and also for expression studies. W, water has been withheld from plants or seedlings; ADP, air-dried plants; ADL, air-dried leaves; DS, desiccated seedlings.

[c] Organ/tissue where the gene is upregulated, after drought treatment.

[d] Expression by application of exogenous ABA in the absence of drought treatment. The ABA-induced expression is occurring in the same organ as in "c." +, ABA induced; −, not ABA induced; M, only moderate response to ABA; NR, no report.

1990). The deduced amino acid sequences of two of these cDNA clones (pcC6-19 and pcC27-04) contain the characteristic sequence motifs (mainly a cluster of serines and a lysine-rich sequence) found in other dehydrated-related genes such as those isolated from cotton (Baker *et al.,* 1988), barley (Close *et al.,* 1989), rice (Mundy and Chua, 1988), and maize (Vilardell *et al.,* 1990), which will be discussed later in this section. The proteins encoded by pcC6-19 and pcC27-04 are extremely hydrophilic.

The promoters of two of the genes, CDeT-27-45 (corresponding to the pcC27-45 clone) and CDeT-6-19 (corresponding to the pcC-19 clone), demonstrate to some extent a different expression pattern in transgenic tobacco and *Arabidopsis,* compared to *Craterostigma.* In *Craterostigma* the two genes are induced in the whole plant by desiccation and/or ABA treatment. However, in transgenic tobacco and *Arabidopsis* they are expressed in seeds. It was hypothesized that in the vegetative tissue of *Craterostigma* the signal transduction pathway from water stress to gene expression requires a gene product which exists only in seeds of higher plants (Michel *et al.,* 1993, 1994; Furini *et al.,* 1996). Moreover, in vegetative tissues of these plants, the induction of expression by ABA is only limited to the early developmental stages (Michel *et al.,* 1993, 1994; Furini *et al.,* 1996).

Another example of a drought-tolerant plant is *Lycopersicon chilense.* This wild tomato, which is a natural inhabitant of arid regions of South America, can grow under severe drought conditions (Rick, 1973). The degree of drought tolerance of *L. chilense* is remarkable. Under severe drought conditions it is the only surviving species in the region. Under the controlled conditions of the growth chamber a 4-month-old *L. chilense* plant, drought stressed for 12 days, ψ_w reduces only -0.15 Mpa (Fig. 2). At this stage the plant does not demonstrate any apparent sign of wilting. This species is therefore an ideal model system for studying drought-induced responses. These studies could ultimately result in identification of the molecular mechanisms of drought tolerance. Drought stress leads to the enhanced synthesis of a large number of polypeptides with different molecular masses in leaves of *L. chilense* (Chen and Tabaeizadeh, 1992a). Interestingly, the synthesis of these drought-induced proteins occurs in different time courses during stress. Some of the proteins are induced during the first few days of stress. The synthesis of another set of proteins are induced later during drought treatment. Many of the newly synthesized proteins have corresponding counterparts among *in vitro* synthesized polypeptides. This demonstrates that the changes in protein synthesis are in part due to increases in corresponding mRNA levels. Some of the drought-induced proteins in *L. chilense* are also induced by heat shock, suggesting that the closure of the stomata during drought stress and the subsequent increase in leaf temperature could be the main factor behind the increase of these proteins. On the other hand, many other drought-induced proteins cannot

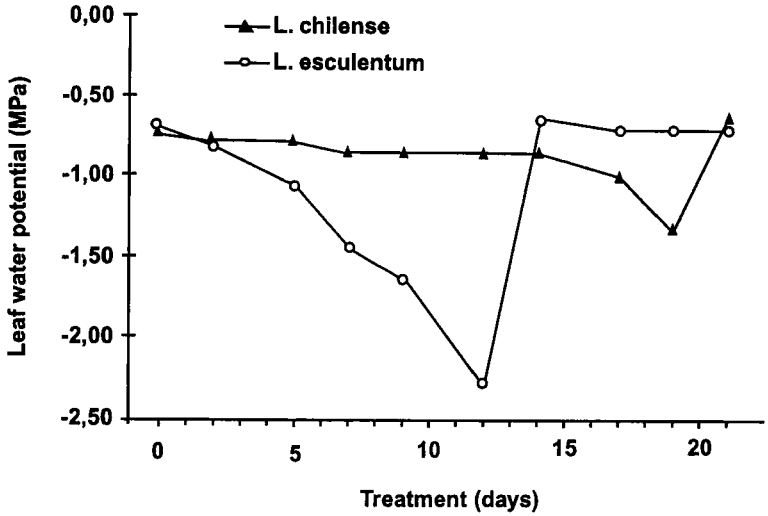

FIG. 2 Leaf water potential of *L. chilense* and *L. esculentum* 4-month-old plants during drought stress and a rehydration period. *L. esculentum* plants have been drought stressed for 12 days and then rewatered up to Day 21, while in the case of *L. chilense,* drought stress has been continued up to Day 19.

be induced by heat shock treatment. Some of the drought-induced proteins in *L. chilense* can also be mimicked by the application of exogenous ABA, demonstrating its involvement in the alteration of gene expression in this species. The role of this plant hormone in regulation of drought-induced gene expression will be discussed further.

Several cDNA clones corresponding to drought- and ABA-induced mRNAs were isolated from *L. chilense* (Chen *et al.,* 1993). The protein encoded by one of these clones (pLC30-15) shares several characteristics in common with some other desiccation- and ABA-induced proteins, including pcC6-19 and pcC27-04 from *C. plantagineum* (Piatkowski *et al.,* 1990), "dehydrins" from barley, maize (Close *et al.,* 1989), and rice (Yamaguchi-Shinozaki *et al.,* 1991); RAB21 from rice (Mundy and Chua, 1988); late embryogenesis abundant proteins from cotton (Baker *et al.,* 1988) and radish (Raynal *et al.,* 1990); and TAS14 from tomato (Godoy *et al.,* 1990). As mentioned earlier, all of these proteins lack cysteine and tryptophan and are overwhelmingly hydrophilic. In addition, these proteins contain several conserved amino acid regions. One is a region containing a cluster of serine residues. The other corresponds to two lysine-rich repeats (designated K boxes).

A study was undertaken in order to establish a phylogenic relationship among these proteins (G. Bellemare and Z. Tabaeizadeh, unpublished

results). Toward this end, the above-mentioned protein sequences were aligned against *L. chilense* pLC30 protein (Devereux *et al.*, 1984; Swofford, 1991). The analysis demonstrated that *L. chilense* pLC30-15 protein belongs to a separate category that diverged early in the course of evolution.

L. chilense is a species that has adapted to arid regions. Rick (1973) has suggested that the adaptation of this species to temperate deserts depends on the foraging ability of its root system. He has proposed that in order to assay the drought tolerance of this species, measuring its reaction to high temperature is required. The pLC30-15 gene is also induced by heat shock. Therefore, it is possible that this protein is at least partly involved in the protection of cellular components under high-temperature stress, which usually occurs in desertic areas.

Two other drought-induced genes have been isolated from *L. chilense;* pcht28, encoding an acidic chitinase (Chen *et al.*, 1994), is noteworthy. This chitinase gene is highly induced in leaves (Fig. 3) and stems by drought stress and ABA. The protein encoded by pcht28 shares 77% amino acid sequence identity with the acidic chitinases PR-P and PR-Q from tobacco (Payne *et al.*, 1990).

The induction of chitinase in *L. chilense* by drought can be considered one of the most common side effects of water stress, i.e., increased susceptibility to pathogens, which has been known for a long time. For example, exposure of tomato plants to low soil moisture for 30 days prior to inoculation with *Fusarium oxysporium* made the plants more susceptible (Foster and Walker, 1947). Other studies suggest that water stress is a very important factor in predisposing plants to severe fungal attacks (Ghaffar and

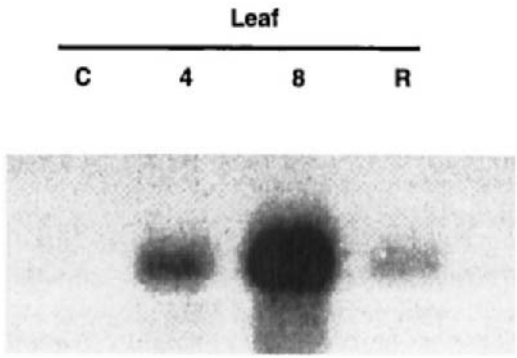

FIG. 3 Chitinase gene expression in *L. chilense* during drought and rehydration. A Northern blot analysis was carried out using RNA isolated from plants watered daily (C), 4- and 8-day drought-stressed plants (4 and 8), and 8-day drought-stressed plants which have been rewatered for 3 days (R). From Yu *et al.* (1997).

Erwin, 1969; Rotem *et al.*, 1968). We hypothesized that the drought induction of chitinase in *L. chilense* is a secondary defense mechanism that this species has evolved in order to protect itself from fungal attack during water stress. However, pcht28 is also induced by exogenous application of ABA (Fig. 4). Therefore, its induction during drought could simply be due to an increase in the endogenous ABA level. Despite of these hypothesizes, we conducted further experiments in order to verify whether this chitinase gene play a role in *L. chilense* drought tolerance.

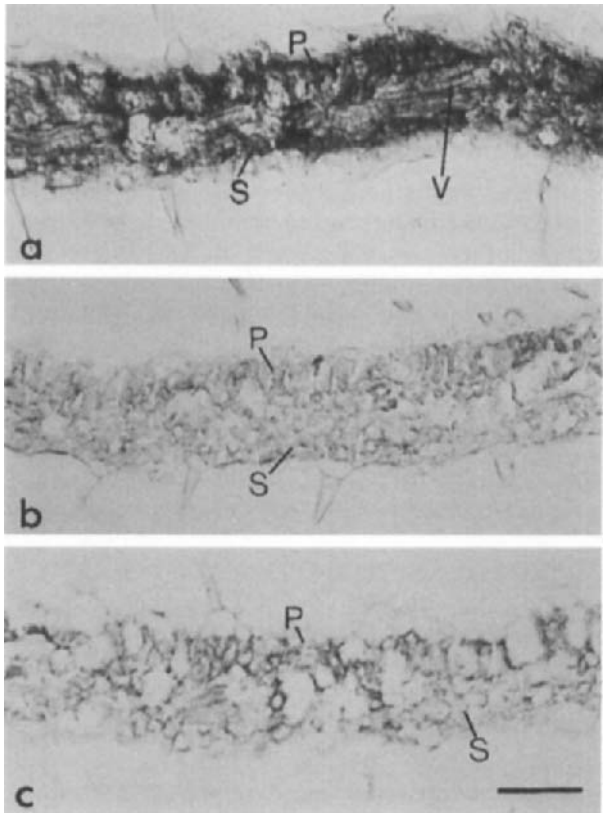

FIG. 4 Localization of pcht mRNAs in *L. chilense* leaves. (a) Cross section of a leaf from a plant submitted to water stress for 8 days. Tissues are disorganized due to desiccation. An intense staining is noted in the palisade and the spongious parenchyma. (b) In this section from a plant watered daily, cells are more easily distinguishable than those in a, and the staining is much lighter. (c) Section from the same specimen as in a. Only weak staining is noted in the absence of the probe in the hybridization mixture. P, palisade parenchyma; S, spongy parenchyma; V, vascular bundle. Bar, 100 μm. From Yu *et al.* (1997).

2. Genes Expressed in Developing Embryos

In higher plants seeds undergo an extreme desiccation during maturation. During the last stage of development the water content of the seed tissues is about 5–10% (Leprince *et al.,* 1993). In early studies (Dure *et al.,* 1981), a group of nonstorage proteins which becomes very abundant during the last period of seed formation was identified in cotton. The genes for many of these proteins designated LEA (for late embryogenesis abundant) were identified (Baker *et al.,* 1988). The expression of these genes correlates with the acquisition of water-stress tolerance in seeds. The accumulation of LEA proteins is largely transcriptionally regulated since their abundance corresponds to the increase in the levels of their mRNAs (Galau *et al.,* 1986, 1987). The expression of some of the LEA genes remains high in seeds at maturity. This is not the case for the genes encoding storage proteins. The transcripts of these proteins start to accumulate to high levels at the same time as LEA transcripts; however, they become undetectable or decline significantly in mature seeds (Galau *et al.,* 1987). The difference in expression of these two classes of genes could be related to the functional differences between their respective proteins. The potential roles of LEA proteins will be discussed in another section.

Each of the identified LEA genes belongs to a small multigene family of two to four members (Baker *et al.,* 1988). The cotton LEA proteins have been classified into five groups (LEA D-7, LEA D-11, LEA D-19, LEA D-95, and LEA D-113) through a dot matrix analysis, based on their extensive homology with at least one protein from another species (Dure *et al.,* 1989; Dure, 1993a). Generally, the concentration of LEA proteins is very high. For example, in mature cotton embryos the level of LEA D-7 and LEA D-113 proteins is about 226–283 μM, respectively, in the cell aqueous phase immediately prior to seed desiccation (Roberts *et al.,* 1993). Interestingly, several genes with extensive homology to LEA genes have been found to be expressed in vegetative tissues (mostly leaves) of plants exposed to water stress. For example, pcC 3-06 and Pc C-6-19 from *C. plantagineum* (Piatkowski *et al.,* 1990), pLC30-15 from *L. chilense* (Chen *et al.,* 1993), B8, B9, and B17 from *Hordeum vulgar* (Close *et al.,* 1989), Em from *Triticum aestivum* (Litts *et al.,* 1987), pRAB16A from *Oryza sativa* (Mundy and Chua, 1988), and pRABT1 from *Arabidopsis thliana* (Lang and Palva, 1992). In addition, desiccation treatment can lead to the precocious expression of LEA proteins in seeds. These findings have led to the assumption that these proteins are involved in drought tolerance.

Regarding the secondary and tertiary structures, several families seem to exist principally as random coils, while others most likely exist as an amphiphilic α-helix (Dure, 1993a). LEA proteins are highly hydrophilic (Baker *et al.,* 1988; Espelund *et al.,* 1992; Dure, 1993a). Therefore, it is unlikely that they play a structural role.

In another study, a water-stress- and ABA-induced cDNA (RAB17) was isolated from a maize embryo (Vilardell *et al.,* 1990). The gene is also inducible in vegetative tissues. The predicted protein of RAB17 contains a cluster of serine residues followed by a consensus site for casein kinase II. Following this domain, there is a basic amino acid sequence, rich in lysine. Similar domains have been found in a mammalian nucleolar phosphoprotein designated Nopp 140 (Meier and Blobel, 1992), which has been identified as a nuclear localization signal (NLS) binding protein that can function in nuclear cytoplasmic transport. Accumulation of RAB17 mRNA in the various tissues of developing embryos has been studied (Goday *et al.,* 1994). During the initial stage of embryogenesis the mRNA is found in the embryo axis; however, during maturation the accumulation occurs in both scutellum and axis cells. Interestingly, during embryo desiccation the outermost tissues (epidermis cells, radical cortex, and coleoptile) show the most significant expression of the gene.

3. Case Studies of Drought-Induced Genes in Crop Plants

There are several reports on isolation of drought-induced genes from crop plants. It is not the purpose of this review to provide details on characterization of all of these genes. Few examples will be highlighted in this section.

Studies on barley and corn seedlings subjected to dehydration led to the identification of five cDNAs (four in barley and one in corn), corresponding to mRNAs which accumulate in response to drought stress. The deduced amino acid sequences of the proteins encoded by these genes are similar to each other. They are extremely hydrophilic and have a high content of glycine. They all contain two lysine-rich repeating units. The mRNAs corresponding to these cDNAs are abundant in dehydrated seedlings, but not in well-watered ones (Close *et al.,* 1989). Later on, several other water-stress-induced cDNAs were isolated from other crop species including wheat (King *et al.,* 1992) and rice (Kusano *et al.,* 1992). A different designation has been assigned to these genes; however, the encoded proteins of these and some other genes discussed earlier (*pLC30-15* from *L. chilense; dsp-14* and *dsp 16* from *C. plantagineum*) are called dehydrines. They are all characterized by the consensus 15-amino-acid sequence domain EKKGIMDKIKEKLPG which is present in most cases at or near the carboxy terminus. It is also repeated upstream of the terminus at least once. Another characteristic which is shared by most of the dehydrins is a tract of serine residues. As mentioned earlier, most of the dehydrines are glycine rich and lack cysteine and tryptophan.

A gene (designated sa/T) which is induced in air-dried or salt-stressed rice seedlings was isolated from a salt-sensitive rice genotype, using a cDNA library prepared from root mRNA (Claes *et al.,* 1990). sa/T mRNA accumu-

lates in sheath and root tissues, but not in the lamina, in salt-treated as well as in air-dried plants. It has been reported that the rice sheath accumulates four times more Na^+ than the leaf laminae (Yeo and Flowers, 1982). The differential expression of sa/T in rice laminae and sheath is similar to the pattern of Na^+ accumulation in these tissues and can be an indication of a coordinated response between the cells of different parts of a stressed plant. The response to both type of stresses, with regard to the expression of sa/T, is very rapid: 3.5 and 6 h for air-dried and salt-treated plants, respectively. This rapid response led to the conclusion that the production of sa/T protein is part of the primary defense or adaptive response and is not symptomatic of damage (Claes *et al.,* 1990). However, the validity of this conclusion can be questioned, since the rice genotype used in this study was salt sensitive. Examination of the expression of this gene in different genotypes having different levels of salt sensitivity could provide more evidence to support this conclusion.

In another study, by using an antiserum raised against maize dehydrin (Close *et al.,* 1989), Robertson and Chandler (1992) isolated a cDNA clone (pPsB12) from a pea cotyledon expression library. The protein encoded by pPsB12, designated pea dehydrin, is rich in glycine and lacks tryptophan. It contains conserved amino acid regions found in several other dehydrins such as those from barley, maize, *C. plantagineum,* and *L. chilense,* which were discussed earlier. The level of pPsB12 mRNA increases significantly in dehydration-stressed seedlings, as is the case for the protein. This accumulation is associated with elevated levels of endogenous ABA.

Another dehydration-induced gene isolated from barley (*paf93*) encodes a protein with a high content of glutamic acid and no cysteine and tryptophan residues, similar to other dehydration-induced proteins (Grossi *et al.,* 1995). The time course of expression of *paf93* mRNA is somewhat different from that of other dehydration-induced genes. This difference cannot be attributed to the experimental conditions since in the same study a probe corresponding to maize and barley dehydrin, Dhn1 (Close *et al.,* 1989), was used. In detached leaves the induction of Dhn1 occurs after 5 h (4.3% loss of fresh weight), whereas the expression of *paf93* can be detected only after 30 min (0.8% loss of fresh weight).

4. Putative Biochemical Function of Drought-Induced Genes

The biochemical function of the drought-induced genes which were explained in this section is not known. The induction of these genes either is as part of a primary adaptive response to reduce damage or is the result of the damage that has occurred. It is logical to believe that the more rapidly a gene is induced the more likely it is related to an adaptive response.

The first step of assessing if a gene product is involved in drought tolerance is to be able to correlate the level of the gene product with the level of stress tolerance. This is especially feasible when within a given species different genotypes with different degrees of tolerance are available. For example, we have studied two different genotypes of *L. chilense,* LA1930 and LA2747. Even though both genotypes can be considered drought tolerant, LA2747 can withstand a longer period of drought. This has also been confirmed through the measurement of different parameters such as leaf water potential, plant growth, etc. during drought stress. When these two genotypes are exposed to drought stress, the drought-induced genes *pLC30-15* and *pcht28* (encoding an acidic chitinase) are expressed at a higher level in LA2747 compared to that in LA1930. This was verified by examining accumulation of mRNA (for both genes) and enzyme activity (for chitinase). Evidently, the expression of these two genes is either very low or undetectable in cultivated tomato (Yu *et al.,* 1997). Another example is the case of the dehydrins pTd27e and pTd25a, isolated from *Triticum durum* (Labhilili *et al.,* 1995). These genes demonstrate different levels of expression in drought-tolerant and drought-sensitive cultivars. In the drought-tolerant cultivar accumulation of dehydrin mRNA is delayed. In the drought-sensitive cultivar, however, the expression is transient, i.e., it disappears later during drought treatment. Moreover, in the drought-tolerant cultivar the level of mRNA accumulation is higher compared to that in the sensitive ones.

In order to verify whether drought-induced genes play a role in the plants' defense against water stress, the encoded protein should be studied directly. The elucidation of the localization of a protein at cellular and subcellular levels can provide more insights on its possible role. This approach has been used for several of the genes which were outlined previously.

Polyclonal antibodies raised against proteins encoded by *C. plantagineum* drought-induced genes, *pcC6-19* and *pcC-04,* were used in Western blot analysis of total protein extracts. The analysis revealed a signal in fully hydrated leaves (Schneider *et al.,* 1993). However, no hybridization signal had been detected when the corresponding clones had been used in Northern blot analysis. This situation was explained by the fact that there is always a possibility that the protein recognized by the antisera in untreated leaves corresponds to a more distant member of the family which is not desiccation induced (Schneider *et al.,* 1993). It is therefore possible that some of these proteins are involved in cellular metabolism under normal conditions. The Western hybridization signal is stronger in dehydrated leaves, which could mean that they are necessary at a higher level during the period of water stress. Immunogold electron microscopy showed that the proteins encoded by *pcC-04* and *pcC6-19* are localized in the cytosol.

Two other proteins encoded by *pcC3-06* and *pcC13-62* are mainly accumulated, respectively, in the stroma and thylakoid membranes of dehydrated leaves (Schneider *et al.,* 1993).

Asghar *et al.* (1994) carried out immunolocalization studies, using antibodies raised against the highly conserved consensus region of plant dehydrins, and a section of maize kernels. Dehydrins were found to be associated with the cytoplasm and nuclei of aleurone, scutellar epithelium, scutellar provascular strands, the outermost embryonic leaves, and in scutellar parenchyma. However, the relative distribution of dehydrins between cytoplasm and nucleus differs among these tissues. Moreover, shoot and root apex cell dehydrins mainly appeared in the cytoplasm. The localization of maize dehydrins in nuclei differs to some extent from that in other studies where dehydrin-related proteins have been found to be localized in the cytoplasm. For example, rice dehydrin (Rab21) was detected only in cytosolic cell fractions (Mundy and Chua, 1988). As mentioned earlier, dsp 16 protein (corresponding to the pcC6-19 cDNA clone) of *C. plantagineum* was localized in the cytoplasm (Schneider *et al.,* 1993). On the other hand, Rab17 from maize embryo was localized in the nucleus and cytoplasm of the sections prepared from the axis and scutellum of embryo cells (Goday *et al.,* 1994). This protein was reported to be phosphorylated at a tract of serine residues (Vilardell *et al.,* 1990). The phosphorylation becomes more intense as the embryo desiccates (Plana *et al.,* 1991). Asghar *et al.* (1994) presented a hypothesis in order to explain the cytoplasm localization of dehydrin homologs (Rab21 and dsp 16 from rice and *C. plantagineum,* respectively) in leaf cells which is different from nuclear localization in maize embryo cells. They used two elements to justify their hypothesis: (a) the existing homology among dehydrins (including Rab17) that contain the serine block and (b) the study of Meier and Blobel (1992) demonstrating that nuclear localization of signal-binding proteins is dependent on the phosphorylation of serine residues. According to Asghar *et al.* (1994), the phosphorylation of serine residues can be the key factor for nuclear localization of dehydrins and it is possible that the dehydrins in rice or *C. plantagineum* leaf cells are not sufficiently phosphorylated in order to be associated with nuclei. However, Rab17 is found in phosphorylated and unphosphorylated forms in both cytoplasm and nuclei (Goday *et al.,* 1994). Moreover, the highest phosphorylated forms of this protein are more abundant in the cytoplasm than in the nucleus. This observation weakens the hypothesis of Asghar *et al.* (1994) regarding the correlation between the phosphorylation state of a protein and its nuclear localization.

As for the possible role of dehydrins, due to their modular segments and their relative abundance in the cells (about 0.5% of soluble protein), it has been suggested that they might have a structural role (Asghar *et al.,* 1994). Their accumulation during dehydration-related environmental stresses and

their presence in the cytoplasm are similar to those of "compatible" solutes such as glycine betaine and sugars (Csonka and Hanson, 1991). Therefore, the structural role (if any) of dehydrins can be related to the stabilization of the cytoplasmic proteins against denaturation, as suggested for compatible solutes (Wyn Jones, 1984; Carpenter and Crowe, 1988; Warr *et al.,* 1988). Stabilization of the nucleoar trafficking apparatus has also been suggested as a possible function for phosphorylated dehydrins (Asghar *et al.,* 1994).

A more founded hypothesis has been presented for Rab17. As mentioned earlier, this protein contains domains common with Nopp 140, which is a nuclear localization signal binding protein. Rab17 can interact with synthetic NLS peptides; however, this binding is dependent upon phosphorylation (Goday *et al.,* 1994); in the unphosphorylated form, it does not interact with NLS peptides. Silver (1991) has suggested that cytoplasmic binding proteins can recognize NLSs and transport proteins to the nuclei. They are then released and recycled in the cytoplasm. Therefore, it is possible that Rab 17 functions as a nuclear import/export carrier (Goday *et al.,* 1994).

A role as a "water-binding protein" has been suggested for the members of the LEA D-19 family (Roberts *et al.,* 1993). This is conceivable considering the hydrophilic nature of these proteins. By binding to water, these proteins can protect the seed from losing all its water, which can indeed be lethal. The hydrodynamic properties of Em protein, which can be considered a LEA D-19 representative from wheat, have been studied (McCubbin *et al.,* 1985). It was shown that the protein is an over 70% random coil under normal physiological conditions. Therefore, it is more hydrated than the most globular polypeptides. Based on this observation, it was suggested that the Em protein prevents total desiccation of the embryo due to its enhanced water-binding capacity. Another group of LEA proteins, the D-7 family, is proposed to protect the cell during dehydration through sequestration of ions. Members of the D-7 family have an 11-mer amino acid motif which is predicted to form an amphiphilic α-helix. It was suggested that the charged side of the protein is involved in ion sequestration in dehydrated cells (Dure, 1993b).

In tomato plants subjected to water stress, a 65-kDa protein (designated TDI-65) accumulates in the leaves and returns to the control level upon rehydration of plants (Chen and Tabaeizadeh, 1992b). Antiserum raised against the purified protein was used in immunofluorescence and immunogold localization studies (Tabaeizadeh *et al.,* 1995). The protein was found to be mainly present in nuclei (peripheral chromatin masses, nucleoli, and nucleoplasm), thylakoid lamella of chloroplasts, and some leaf cell cytoplasmic regions. Quantification of the gold labeling clearly showed significant differences in the distribution of the protein between plants subjected to water stress and control plants (Table V; Figs. 5 and 6). The labeling density of heterochromatin masses was especially striking. It was suggested

TABLE V

Quantitative Evaluation of the Gold Labeling (Gold Particles/μm^2) Detected in Tomato Cell Nuclei and Chloroplasts Using Antiserum Raised against the Tomato Drought-Induced Protein TDI-65 (from Tabaeizadeh *et al.*, 1995)

	Cellular compartments		
	Nuclei		
	HC[a]	IC[a]	Chloroplast
Treated plants	536.1 ± 32.7	162.0 ± 18.7	225.2 ± 17.6
Control plants	68.1 ± 5.5	49.5 ± 3.4	35.7 ± 4.2

Note. The differences in labeling density over nuclear HC and IC areas as well as over chloroplasts were significant ($p < 0.0001$) between 12-day drought-stressed and control plants. Differences in labeling density between HC and IC areas within the same group of samples were also significant ($p < 0.01$).

[a] HC, heterochromatin masses; IC, interchromatin areas (including fibrillogranular aggregates and more loosened material).

that TDI-65 protein may play a protective role in maintaining the ultrastructural integrity of nuclei and/or chloroplasts in the cells subjected to dehydration. Moreover, its significant association with the chromatin strongly suggests a role in maintaining the integrity of DNA. Isolation of the gene encoding TDI-65 protein can certainly be of significant value to elucidate its nature and its biochemical function.

5. Assessment of Roles of Drought-Induced Genes through Gene Transfer Techniques

Even though the function of water-stress-induced genes is not clear, their role can be assessed through transformation techniques. The inhibition of gene expression by transformation with antisense constructs can be considered one of the approaches in order to verify if a gene plays a role in drought tolerance. The antisense strategy has been used for almost a decade to inhibit the expression of specific genes (Delauney *et al.*, 1988). Theoretically, if the accumulation of a drought-induced gene product can be inhibited or reduced at a significant level, any reduction in the drought tolerance of the resulting transgenic plants can be attributed to that gene. However, this approach does not seem to be suitable for drought-induced genes since in almost all cases, these genes belong to small families. Therefore, the inhibition of one single gene will not lead to a significant reduction of the gene product.

The dehydration-induced genes can be overexpressed, in order to assess if their products have a role in drought tolerance. Ideally, the plant species

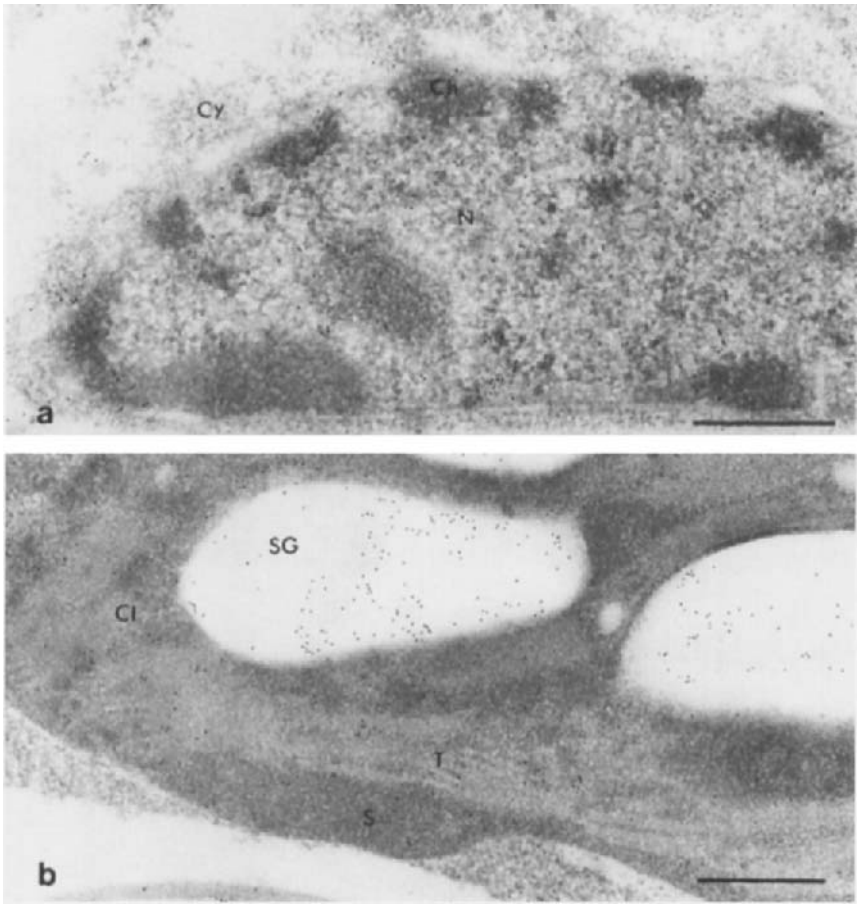

FIG. 5 Immunogold localization of the drought-induced protein TDI-65 in leaf mesophyll cells of tomato (*L. esculentum*) plants which have been watered daily. (a) Portion of a nucleus (N) showing a few scattered gold particles that appear to be only slightly more concentrated over the chromatin masses (Ch). The cell cytoplasm (Cy) is nearly devoid of labeling. Original magnification, ×53,650. Bar, 0.5 μm. (b) In the case of chloroplasts (Cl), a few gold particles are noted over the stroma (S) and thylakoid lamellae (T), the labeling being more intense over the starch grains (SG). The cytoplasm (Cy) is unlabeled. Original magnification, ×47,250. Bar, 0.5 μm. From Tabaeizadeh *et al.* (1995).

to which the gene is transferred should be the one from which the gene has been isolated. This is especially important since there is always the possibility that the gene product does not function in another cellular background the way it does in its native cellular environment. However, those species from which drought-induced genes have been isolated are

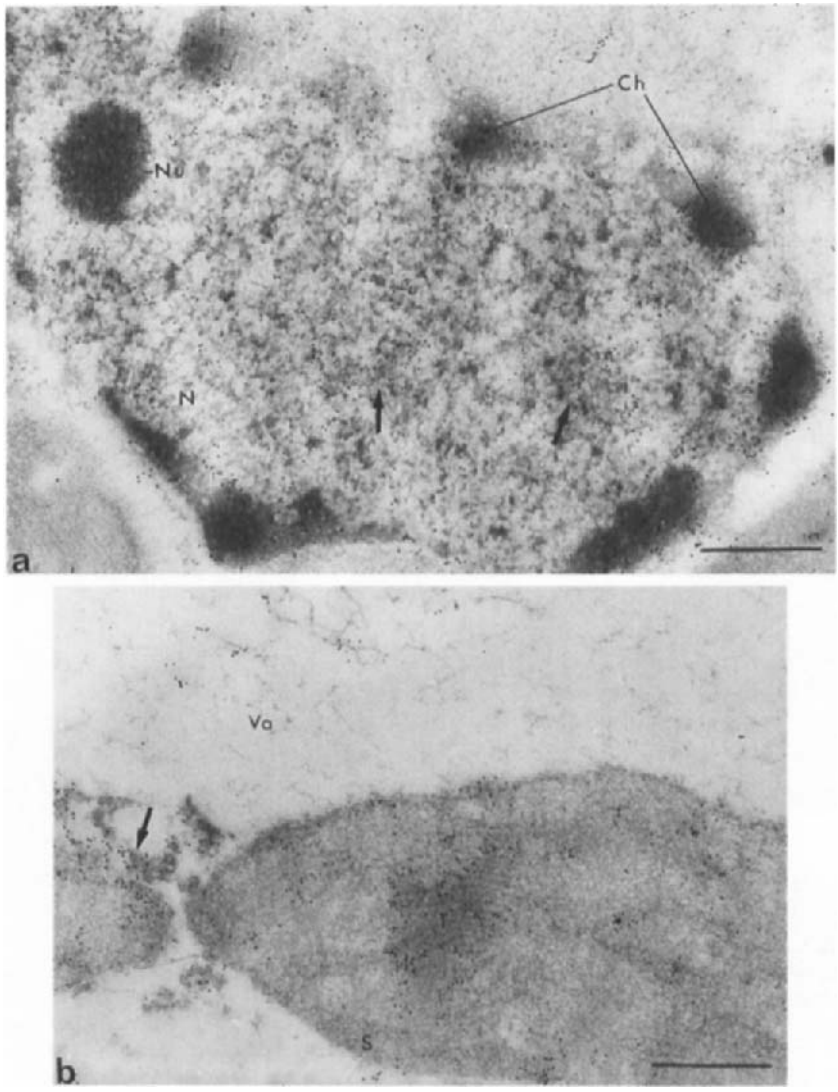

FIG. 6 Immunogold localization of the drought-induced protein TDI-65 in leaf mesophyll cells of tomato (*L. esculentum*) plants drought stressed for 12 days. (a) Numerous gold particles are present over the peripheral chromatin masses (Ch), with the nucleolus (Nu) and the fibrillogranular nucleoplasmic material (arrows) being less intensely labeled. N, nucleus. Original magnification, ×47,250. Bar, 0.5 μm. (b) A portion of cytoplasm from same material illustrated in a. In this portion of chloroplast, gold particles are principally concentrated over regions corresponding to thylakoid lamellae (T) but a few gold particles are also scattered over the stroma (S). Cytoplasmic fragments (arrow) and fibrillar-like material in the vacuole (Va) also display some labeling. Original magnification, ×47,250. Bar, 0.5 μm. From Tabaeizadeh *et al.* (1995).

not always amenable to transformation and regeneration techniques. In some cases tobacco has been used, mostly due to the fact that it is very easy to transform.

Two of the *C. plantagineum* drought-induced genes (*pcC6-19* and *pcC3-06*) were transferred to tobacco. Transgenic plants constitutively expressing *pcC-6-19* or *pcC3-06* did not show any improvement in drought tolerance when measured by an ion-leakage test or in photosynthetic ability (Iturriaga *et al.,* 1992). This is not very surprising, since drought tolerance is a much more complicated mechanism than can be expected to be improved by expression of a single protein. An ideal case, therefore, would be to overexpress simultaneously several of these genes to a plant species and then examine the reaction of transgenic plants to drought stress.

An efficient transformation–regeneration technique was developed for *L. chilense* (Agharbaoui *et al.,* 1995). As mentioned earlier, one of the drought-induced genes (*pcht28*), which was isolated and characterized from this species, encodes an acidic chitinase. We took advantage of the known role of chitinase and introduced *pcht28* to cultivated tomato, resulting in production of plants which are resistant to *Verticillium dahliae* (Tabaeizadeh *et al.,* 1997). Moreover, we were anxious to determine if there is a slight possibility that this gene plays a role in drought tolerance. We became inspired by the observed correlation between the degree of drought tolerance and the expression of *pcht28* in the two *L. chilense* genotypes, LA2747 and LA1930. For each genotype we have produced transgenic plants in which *pcht28* is constitutively overexpressed (M. Djebrouni and Z. Tabaeizadeh, unpublished results). Once the insertion of the transferred gene was confirmed, the transgenic plants were analyzed under normal and drought-stressed conditions, with regard to leaf water potential, accumulation of pcht28mRNA, and chitinase enzymatic activity. Our results, yet preliminary, but the fruit of extensive studies, are encouraging. As expected, the transgenic plants, growing under normal conditions, demonstrate a high level of constitutive expression of the chitinase gene. Under the same conditions, chitinase gene expression is undetectable or at a very low level in nontransformed *L. chilense.* When the transgenic plants are drought stressed, there is a cumulative (constitutive plus induced) expression of the gene, which is also expected. We have identified several transgenic individuals in which chitinase gene expression is extremely high compared to that in drought-stressed untransformed plants. Remarkably, upon casual observation, these plants seem to be more tolerant to drought stress compared to nontransformed plants. Furthermore, during drought stress the transgenic plants are capable of maintaining a significantly higher leaf water potential compared to that in nontransgenic plants. We have used enough replicates for each individual to make sure that the observed results are not due to phenotypical variation, if any, among the plants. Measurement

of other parameters such as biomass and photosynthetic activity will allow us to draw a conclusion on the possible role of this chitinase in drought tolerance of *L. chilense.*

6. Regulation of Water-Stress-Induced Genes; Role of Abscisic Acid

The plant hormone abscisic acid plays different roles in higher plants. The biosynthesis and metabolism of ABA have been the subject of numerous studies (Walton, 1980; Zeevaart and Creelman, 1988). Two pathways have been proposed for ABA biosynthesis. The first is a direct pathway involving a C_{15} precursor derived from farnesyl pyrophosphate. The second is an indirect pathway which involves a precursor derived from a cartenoid (Zeevaart and Creelman, 1988). Different experimental evidences suggest that the indirect pathway is dominant in higher plants. The inhibition of ABA accumulation by inhibitors of cartenoid biosynthesis, such as fluridone, can be accounted for as strong evidence in favor of the indirect pathway (Moore and Smith, 1984; Zeevaart and Creelman, 1988).

It is well documented that a number of plants' physiological and developmental processes are mediated by abscisic acid (Giraudat *et al.,* 1994). For example, during the late period of embryogenesis, the level of endogenous ABA increases and simultaneously specific mRNA and proteins accumulate in cells. This is the time when the seeds desiccate and the embryos of some plant species become dormant (King, 1982; Galau *et al.,* 1986). The specific mRNA and proteins are usually rapidly degraded during seed germination; however, they can reappear in germinating seeds by application of ABA (Finkelstein *et al.,* 1985; Mundy *et al.,* 1986).

Early studies have shown that endogenous ABA also increases in vegetative tissues of plants subjected to water stress. The increase in the endogenous level of ABA during water stress has been suggested to be caused by *de novo* synthesis of the hormone. Some other studies propose the release of ABA sequestered in organelles (Zeevaart and Creelman, 1988). It has also been suggested that during water stress a set of genes is induced by loss of turger pressure, which in turn regulates the ABA level (Guerrero and Mullet, 1988). Once the ABA level increases, the expression of specific genes is induced. In most cases, these genes, which are thought to have a protective function, can also be induced by application of exogenous ABA in the absence of stress. Numerous studies have been carried out in order to understand the regulation of gene expression by ABA during water stress. An elegant example is the study involving isolation and characterization of a gene, *Rab21,* whose expression is induced by water stress and ABA in rice (Mundy and Chua, 1988). The level of *Rab21* mRNA is very low in the embryo, during the early period of seed formation; however, it

significantly increases during the period between 20 days after flowering and maturity. Moreover, *Rab21* mRNA survives as long-lived mRNA in the resting grain but is rapidly turned over at the onset of germination. When the germinating embryos are incubated in 10 μM ABA, this mRNA again accumulates. This is clearly demonstrating that ABA affects the steady-state level of *Rab21* mRNA. The mRNA is not detectable in the roots and shoots of hydroponically grown rice plants; however, when ABA is added to the medium it accumulates in both organs. Addition of NaCl also leads to the accumulation of *Rab21* mRNA, interestingly, with a time course of induction which is similar to that for ABA treatment. When both ABA and NaCl are added to the hydroponic solution, the pattern of *Rab21* mRNA accumulation remains unchanged, meaning that the effects of these two inducers are not cumulative. This can suggest that ABA and NaCl have a common response pathway (Mundy and Chua, 1988). The induction of *Rab21* by ABA is not dependent on *de novo* protein synthesis. This was demonstrated by treating the cell suspension cultures in which ABA normally induces the accumulation of the mRNA after about 15 min with protein synthesis inhibitors which reduce total protein synthesis by >90%. These inhibitors did not block the induction of *Rab21* mRNA by ABA. It was suggested that the ABA induction of gene expression is mediated by preexisting factors (Mundy and Chua, 1988). Indeed, this has been shown for the heat shock response (Zimarino and Wu, 1987).

The analysis of published results associates, in most cases, the expression of dehydration-induced genes with ABA. This is supported by the fact that (a) in the absence of stress, the treatment of the tissue with ABA leads to the expression of the genes which are normally induced by dehydration, (b) expression of water-stressed-induced genes is accompanied by an increase in the endogenous (cytosolic and apoplastic) ABA level, and (c) under water stress conditions, mutants which do not synthesize ABA (probably due to the blockage in their cartenoid biosynthetic pathway) do not express the genes which are induced in their respective isogenic lines in response to dehydration. However, there are also some evidences which may suggest that ABA is not the principal regulator of water-stress-induced genes. For example, the expression of dehydrin genes in barley aleurone can be induced by phaseic acid (Chandler and Robertson, 1994), the first major metabolite of ABA. The cellular level of gibberellic acid can also influence the expression of dehydrin genes by ABA. In barley aleurone, gibberellic acid inhibits the expression of dehydrin in response to ABA (Chandler and Robertson, 1994).

The ABA regulation of dehydration-induced gene expression has been studied using mutants with reduced levels of endogenous ABA. For example desiccated leaves of a tomato ABA deficient mutant *flacca* do not express the genes which are induced in the desiccated leaves of the isogenic

line Ailsa (Bray, 1988). However, these genes can be expressed in the mutant line upon treatment with ABA (Cohen and Bray, 1990). The amount of ABA necessary for the induction of these genes in *flacca* is lower than that for Ailsa.

The involvement of ABA in regulation of water-stress-induced genes can be at transcriptinal or posttranscriptional levels. For example, ABA treatment of *L. chilense* plants led to an increase in chitinase (*pcht28*) mRNA and enzyme activity (Chen *et al.*, 1994; Yu *et al.*, 1997). On the other hand, the expression of osmotin mRNA in tobacco leaves or cultured cells is significantly induced by ABA, whereas no effect of ABA can be detected on accumulation of osmotin protein (LaRosa *et al.*, 1992).

The ABA regulation of dehydration-related genes can be organ specific and can vary according to developmental stages (Pla *et al.*, 1984). The studies with viviparous mutants of corn can be cited as an example. In these mutants the level of ABA in the developing embryos and in water-stressed seedlings is very low (Neill *et al.*, 1986). The gene *rab28* is expressed in the developing embryos but not in dehydration-stressed seedlings (Pla *et al.*, 1991). This clearly demonstrates that ABA is not required for the expression of *rab28* in developing embryos.

ABA regulation of water-stress-induced genes can vary significantly in different types of cells. For example, Rab17 is known to be induced upon ABA treatment in the maize embryo. This induction is more pronounced in scutellum compared to that in the axis cells. Moreover, ABA treatment cause a decrease in mRNA levels in mesocotyl and provascular cells (Goday *et al.*, 1994). This observation can demonstrate the existence of different mechanisms behind the expression of the gene. However, other possibilities may exist as well. For instance, as stated, the distribution of ABA may not be uniform in all cell types, even though they are apparently all subjected to ABA treatment. Another explanation could be that the stability of Rab mRNA is not the same in different cell types. Goday *et al.* (1994) suggested that the decrease in Rab mRNA is due to a rehydration response of the cells upon imbibition in the ABA solution.

There are also reports on dehydration-related genes which cannot be induced by ABA in the absence of stress (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992; Grossi *et al.*, 1992). However, one should take into account the fact that the results of the experiments regarding the expression of dehydration-induced genes in response to exogenous ABA can be largely influenced by the efficiency of ABA uptake. Therefore, the measurement of the endogenous level of ABA in these experiments is of great value. In pea seedlings, for example, high concentrations of exogenous ABA are necessary to induce a low level (compared to water stress condition) of dehydrin. It was revealed that the endogenous ABA level in the ABA-

treated seedlings is less than that in water-stressed seedlings (Robertson and Chandler, 1992).

By measuring the endogenous level of ABA, strong evidences have been presented which suggest that the expression of some of the dehydration-related genes is either ABA independent or triggered by a small increase in ABA content. For example, under the same experimental conditions, in the detached barley leaves, the expression of Dhn1 (Close *et al.,* 1989) is induced after 5 h, when the ABA level has increased to 466 ng/g fresh weight, whereas the expression of paf93 (Grossi *et al.,* 1995) is induced only after 30 min. At this time the endogenous ABA level is 20.5 ng/g fresh weight (Grossi *et al.,* 1995). These results lead to the conclusion that different signal transduction pathways are involved in modulation of plant response to water stress.

The model studies with the tolerant species *C. plantagineum* suggest the involvement of ABA in the resistance response. As mentioned earlier, this resurrection plant can withstand complete dehydration. The desiccation-intolerant calli of this plant can be resurrected after drying if they are pretreated with ABA. During drought stress, the plant itself expresses genes which are ABA responsive. Moreover, the expression of these genes is absent in desiccation-intolerant calli but present in desiccation-tolerant ones (Bartels *et al.,* 1990). The other drought tolerant species, *L. chilense,* also expresses ABA-responsive genes in both leaf tissues of plants subjected to drought stress and cell suspensions subjected to osmotic stress (Chen and Tabaeizadeh, 1992a).

There are also experimental evidences suggesting that ABA is not the only factor behind the regulation of water-stress-induced genes, even in those cases where applied ABA can upregulate the genes under nonstressed conditions. In tomato plants subjected to different types of treatments (desiccation, NaCl, PEG, low temperature, and heat shock) which lead to water stress, three genes, *pLE4, pLE16,* and *pLE25,* are induced. However, the level of expression of these genes does not always correlate with the endogenous ABA level. For example, desiccation treatment leads to a fourfold increase in the endogenous ABA levels and the high expression of the three genes (Cohen *et al.,* 1991; Plant *et al.,* 1991). On the other hand, plants treated with PEG demonstrated very low expression of *pLE4* and *pLE25* despite of the fact that the ABA levels in these plants were again fourfold higher than those in control plants. Interestingly, the expression of *pLE16* was higher in the PEG-treated plants compared to that in desiccated leaves.

From four cDNA clones corresponding to *A. thaliana* root transcripts regulated by drought, three (*AtDr8, AtDr19,* and *AtDr21*) were positively regulated (Gosti *et al.,* 1995). By using ABA treatment and an ABA-deficient mutant the involvement of this hormone in the regulation of

two of these genes (*AtDr8* and *AtDr21*) was clearly demonstrated. The expression of the third gene, *AtDr19,* during drought was ABA independent. The forth clone, *AtDi4,* which will be detailed in another section since it is downregulated by drought, also responded to drought in an ABA-independent manner. However, the response of these two last genes is at different thresholds of progressive drought stress. Based on these results it was suggested that the regulation of these four genes is mediated by three signals, and only one of them is ABA (Gosti *et al.,* 1995).

Some genes are induced by ABA, but their dehydration-induced expression is ABA independent. The kinase gene *RPK1,* isolated from *A. thaliana,* can be cited as an example. This gene is upregulated by dehydration as well as treatment with ABA (Hong *et al.,* 1997). When dehydration responsiveness of *RPK1* was examined in ABA-deficient (aba-1) and ABA-insensitive mutants (abi1-1, abi2-1, and abi3-1), the same levels of mRNA accumulation were observed in the mutants as in the wild type. The hypothesis regarding the ABA-independent dehydration-induced expression of *RPK1* is supported by the fact that in *Arabidopsis* the level of ABA is just detectable 2 h after dehydration (Yamaguch-Shinozaki and Shinozaki, 1993), while *RPK1* is highly induced only within 1 h of dehydration treatment.

Some of the studies regarding the regulation of water-stress-induced genes by ABA have focused on *cis* and *trans* factors, where a *trans*-acting protein factor interacts with an element (*cis*-acting factor) in the promoter region of the gene. A conserved sequence motif, CACGTG, which functions as an ABA-responsive element (ABRE) and modulates the induction of expression of a reporter gene, has been identified (Marcotte *et al.,* 1988, 1989; Mundy *et al.,* 1990; Vilardell *et al.,* 1991; Pla *et al.,* 1993). Plant nuclear extracts have binding activities with specificity for the G box (Williams *et al.,* 1992). The conserved sequence motif in the Rab28 (Gomez *et al.,* 1988; Pla *et al.,* 1993) promoter binds nuclear protein factors from mature embryos and water-stressed leaves (Pla *et al.,* 1993). This demonstrates the involvement of transcriptional controls in ABA regulation. However, binding reactions with the nuclear protein extracts from embryo and water-stressed leaves generate protein–DNA complexes which are different from each other with regard to electrophoretic mobility. In fact, the complexes formed with leaf protein have a slightly increased migration rate compared to those with embryo proteins. A different line of experiments provided enough evidence to suggest that this difference in electrophoretic mobility is due to the fact that different proteins with similar affinity to the ABRE sequence are present in these two type of tissues (Pla *et al.,* 1993). There are some evidences indicating the association of the G box core of the ABRE element in regulation of water-stress-induced genes. For example, slightly different (2 bp per 10-bp repeat) *cis*-acting sequences containing a G box core exhib-

ited different temporal and spatial promoter activity patterns in transgenic tobacco (Salinas *et al.*, 1992).

Studies on the regulatory regions of the drought induced gene *rd29A* of *A. thaliana* have demonstrated the existence of ABA-independent signal transduction pathways which are involved in gene expression. Two *cis*-acting elements were identified in this gene (Yamaguchi-Shinozaki and Shinozaki, 1993, 1994). The first, which functions in the early rapid response of the gene to drought, salt, or low temperature, is a 9-bp direct repeat sequence (TACCGACAT), designated the dehydration-responsive element. The second, however, contains an ABRE.

Several cDNA clones encoding proteins which interact with sequences containing a CACGTG core have been isolated. The detailed description of these proteins does not concern this review; however, it is important to mention that all of these proteins contain a basic domain with leucine repeats. They are classified as basic/leucine zipper transcription factors.

7. Negative Regulation of Gene Expression by Drought Stress; Relation to Abscisic Acid

The plant response to water stress also includes downregulation of specific genes. In dehydrated tomato leaves the expression of *cab* and a Rubisco small subunit gene, *rbcS,* is significantly reduced. This reduction of gene expression is accompanied by an increase in the endogenous ABA level (Bartholomew *et al.*, 1991), demonstrating that the negative regulation of these genes is mediated by ABA. The expression of these genes is also reduced in nonstressed plants upon application of ABA.

A cDNA clone (*AtDr4*) downregulated by drought was isolated from *A. thliana* (Gosti *et al.*, 1995). The drought-induced negative expression of *AtDr4* is not modulated by ABA. *AtDr4* mRNA is expressed in a root-specific manner in regularly watered plants; however, it is undetectable during drought stress. The deduced amino acid sequences of *AtDr4* presents a considerable degree of similarity with those of different seed-specific Künitz protease inhibitors. This is interesting, since the *AtDr4* mRNA is not expressed in *A. thaliana* seeds. This is the first report on a drought-regulated protease inhibitor. There are at least two other genes encoding putative proteases which are positively regulated by drought (Guerrero *et al.*, 1990; Koizumi *et al.*, 1993). Gosti *et al.* (1995) suggested that the biochemical response of plants to drought stress might involve specific changes in proteolytic activities.

In another study involving *L. chilense,* a gene (designated *PTGRP*) which is downregulated by drought was isolated and characterized (Yu *et al.*, 1996). The primary structure of PTGRP protein has some similarities with that of some of the cell wall proteins; high proline content (26%) and

repeated motifs are among the most striking similarities. The expression of *PTGRP* decreases 10-fold in the leaves of 8-day drought-stressed plants. This reduction of expression was also observed in *L. chilense* cell suspensions treated with mannitol, NaCl, demonstrating the downregulation of the gene by osmotic stress. Application of ABA to the nonstressed plants or cell suspensions also led to a significant reduction of *PTGRP* expression. Based on the existing similarity between the primary structure of PTGRP protein and that of some cell wall proteins, it was hypothesized that PTGRP is a cell wall protein and that its downregulation by drought is related to the remodeling of the plant cell wall in response to drought stress. Negative expression of cell wall proteins during other stress situations or by an elicitor has been reported. For example, a gene encoding for a hydroxyproline-rich glycoprotein (Hyp 2.11) isolated from bean is downregulated by elicitor treatments (Sauer *et al.,* 1990). The exact function of these downregulated genes is unknown. However, as mentioned earlier, genes with a known function in photosynthesis such as the structural gene *cab* and the Rubisco small subunit gene *rbcS* are downregulated during dehydration (Bartholomew *et al.,* 1991). This is among some other changes, including closure of the stomata, which lead to a reduction in photosynthetic activity. Therefore, the hypothesis suggesting that some of the genes are downregulated due to the fact that their product might not be suited for the new physiological condition caused by drought stress (Chandler and Robertson, 1994) is logical. Sauer *et al.* (1990) proposed that the dynamic remodeling of the cell wall, in response to pathogen attack, involves two coordinated processes: one, the activation of those genes whose products may be useful for defense, and the other, the deactivation of the genes whose products are more appropriate for the function of the cell wall under normal growth conditions. The same hypothesis can be applied for the response of the plant cell wall under water stress conditions. From the two cell wall proteins identified in soybean seedlings, one is increased during drought stress, while the other is decreased (Bozarth *et al.,* 1987). It is interesting to mention that when the drought-stressed *L. chilense* plants were rewatered, they demonstrated a higher degree of expression of *PTGRP* compared to that in the control plants. This can suggest that besides playing a role in plant growth and development during normal conditions, the *PTGRP* gene product has an important function in plant recovery from drought stress (Yu *et al.,* 1996).

V. Signal Transduction Pathways Involved in the Plant Cell Response to Water Stress

Some of the studies on drought-induced genes such as *Rab17* (Vilardell *et al.,* 1990; Goday *et al.,* 1994) and tomato dehydrin (Godoy *et al.,* 1994)

suggest the involvement of protein phosphorylation in the signal transduction pathway of the cellular response to water stress. This idea is supported by several reports indicating the dehydration-induced expression of genes encoding kinases. As an example, kinase genes isolated from *A. thliana* (Hwang and Goodman, 1995; Mizoguchi *et al.*, 1996) and wheat (Holappa and Walker-Simmons, 1995) can be cited. All of these kinases are upregulated by water stress. Judging from their structure, these kinases seem to be intracellular proteins. More recently, a receptor-like protein kinase gene, *RPK1,* which is also upregulated by water stress, was isolated from *A. thliana* (Hong *et al.*, 1997). The receptor kinases which are associated with the membrane are well characterized in animal cells. It is believed that many of the extracellular signals are first perceived by these receptor kinases (Lemmon and Schlessinger, 1994). Protein kinases with structures similar to those of animal cells have been characterized in a plant system (Walker, 1994; Stone and Walker, 1995). *RPK1* presents the structural features of a transmembrane protein kinase, with an intracellular kinase domain and an extracellular ligand domain. Based on the structural similarity between *RPK1* and animal receptor kinases, Hong *et al.* (1997) suggested that this protein may function in the transmission of environmental stress signals into intracellular target molecules.

Calcium also seems to play a role. The ABA-induced stomatal closure during drought stress is thought to be mediated by a calcium-dependent signal transduction pathway. Several independent studies have shown that ABA induces the elevation of calcium in guard cells (McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990; Allan *et al.*, 1994). Cytoplasmic calcium can be increased in part by release from intracellular stores such as vacuoles and endoplasmic reticulum (Bush, 1995). The increase in the guard cell cytoplasmic calcium concentration activates anion channels in the plasma membrane (Schroeder and Hagiwara, 1989), which in turn leads to stomatal closure (Schroeder, 1995).

VI. Concluding Remarks

Plants should employ a complex array of processes in order to adapt to water stress. This adaptive response is controlled at the level of gene expression. Extensive studies have been carried out on the regulation aspect of drought-induced genes. The most challenging research, yet ahead, is the elucidation of the biochemical role of these gene products. The conserved regions among these genes can be attributed to their functional significance. The fact that specific identical sequences which have been conserved during the evolution of genes which are upregulated by water stress in a wide range

of plant species is very interesting. It can strongly suggest that the conserved sequences of these proteins may correspond to essential domains of their tertiary structures and are critical to their function in the plant's response to water stress. The results on the production of transgenic plants, accumulating one of the osmoprotectant compounds, are encouraging, although they should be studied carefully. A large number of transgenic individuals should be tested before drawing any conclusion. Once the results are confirmed, these genes can be tested in important crop species, such as cereals, for which regeneration–transformation techniques are now available.

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Peptidergic Control of the Corpus Cardiacum–Corpora Allata Complex of Locusts

Dirk Veelaert, Liliane Schoofs, and Arnold De Loof
Laboratory for Developmental Biology and Molecular Biology,
Zoological Institute, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

The brain–corpora cardiaca–corpora allata complex of insects is the physiological equivalent of the brain–hypophysis axis of vertebrates. In locusts there is only one corpus cardiacum as a result of fusion, while most other insect species have a pair of such glands. Like the pituitary of vertebrates, the corpus cardiacum consists of a glandular lobe and a neurohemal lobe. The glandular lobe synthesizes and releases adipokinetic hormones. In the neurohemal part many peptide hormones, which are produced in neurosecretory cells in the brain, are released into the hemolymph. The corpora allata, which have no counterpart in vertebrates, synthesize and release juvenile hormones. The control of the locust corpus cardiacum–corpora allata complex appears to be very complex. Numerous brain factors have been reported to have an effect on biosynthesis and release of juvenile hormone or adipokinetic hormone. Many neuropeptides are present in nerves projecting from the brain into the corpora cardiaca–corpora allata complex, the most important ones being neuroparsins, ovary maturating parsin, insulin-related peptide, diuretic peptide, tachykinins, FLRFamides, FXPRLamides, accessory gland myotropin I, crustacean cardioactive peptide, and schistostatins. In this paper, the cellular distribution, posttranslational processing, peptide–receptor interaction, and inactivation of these peptides are reviewed. In addition, the signal transduction pathways in the release of adipokinetic hormone and juvenile hormone from, respectively, the corpora cardiaca and corpora allata are discussed.

KEY WORDS: Neuropeptide, Corpus allatum, Juvenile hormone, Corpus cardiacum, Adipokinetic hormone, *Locusta migratoria, Schistocerca gregaria.*

I. Introduction

The master endocrine gland of vertebrates is the brain–pituitary complex. Hormones such as oxytocin and vasopressin are produced in the hypothala-

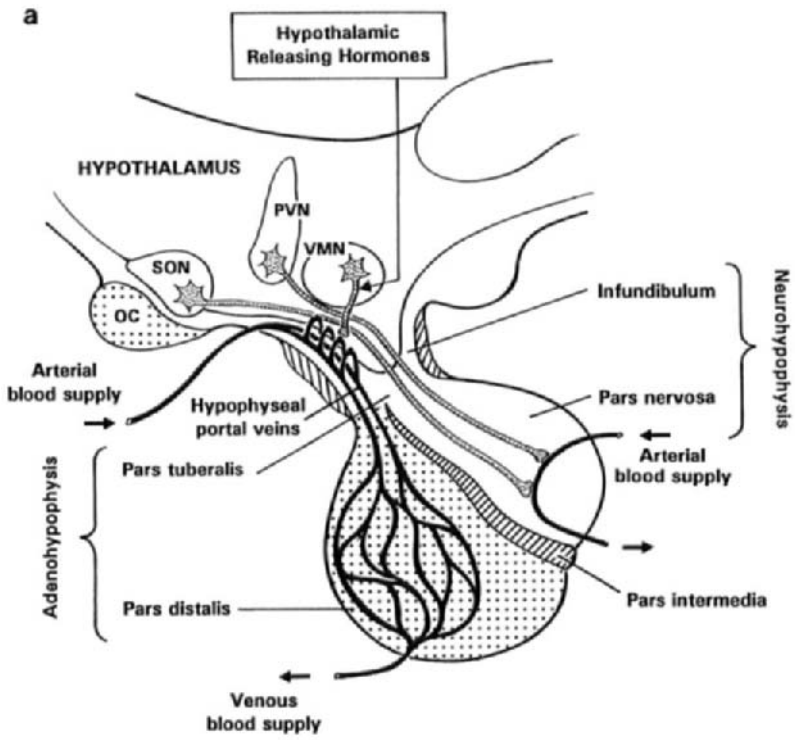

FIG. 1 Comparison of the brain–pituitary complex of vertebrates and the brain–corpora cardiaca–corpora allata complex of insects. (a) Vertebrates: the connections between the hypothalamus and the hypophysis. The hypothalamic releasing hormones are secreted by the neurosecretory cells in the ventromedial nucleus (VMN) and other areas of the hypothalamus. The hypophyseal portal veins carry the hypothalamic hormones to the adenohypophysis. The neurosecretory cells in the supraoptic (SON) and paraventricular nuclei (PVN) project their axons to the pars nervosa (neuohypophysis). Here, the hormones are released in the blood. OC, optic chiasm. (b) Insects: the connections between the brain and the corpus cardiacum complex. Neurosecretory cells in the brain and suboesophageal ganglion (SOG) project their axons via three paired nerves to the corpus cardiacum–corpora allata complex. NCCI, II, III: nervus corporis cardiaci I, II, III; NCA I, II: nervus corporis allati I, II. SOG, suboesophageal ganglion.

mus, transported through axons to the caudal part of the hypophysis (neurohypophysis), and from there are released into the bloodstream (Fig. 1a). The more rostral part of the hypophysis is the glandular part or adenohypophysis (adenos: gland). It consists of the pars distalis (anterior lobe) and the pars intermedia (intermediate lobe). The former synthesizes growth hormone, prolactin, follicle-stimulating hormone, luteinizing hormone, thyroid stimulating hormone, and adrenocorticotrophic hormone, whereas the lat-

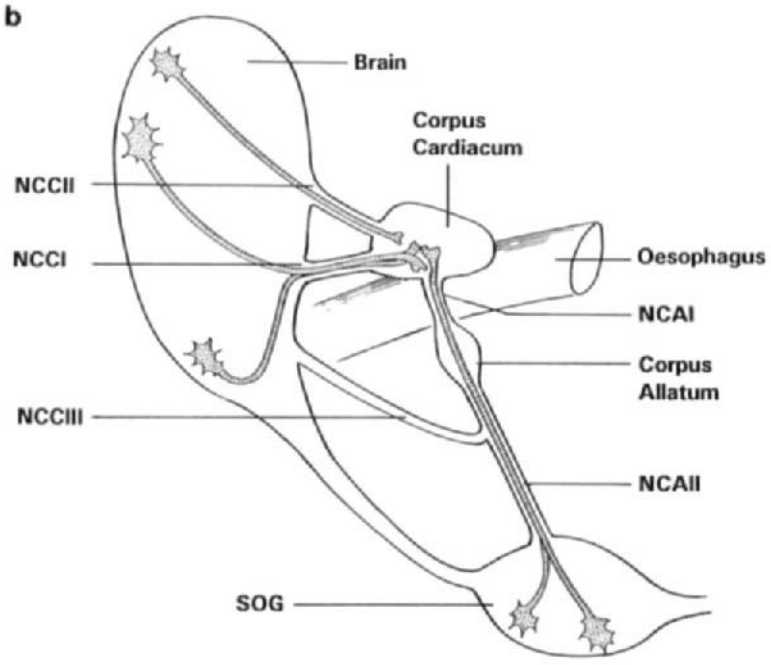

FIG. 1 (*continued*)

ter produces melanocyte-stimulating hormone. The release of these hormones is mainly controlled by releasing and inhibiting factors that are synthesized in the hypothalamus and transported through a portal vein system to the adenohypophysis. The identification of releasing factors was a milestone in vertebrate endocrinology, awarded with a Nobel prize in medicine in 1977 (R. Guillemin and A. Schally).

The brain–corpora cardiaca (CC)–corpora allata (CA) complex of insects is the physiological equivalent of the brain–pituitary axis of vertebrates. However, there are differences which are not at all surprising if one takes into account that the Deuterostomians, to which the vertebrates and echinoderms belong, and the Protostomians, which comprise artrhopods, molluscs, annelids, etc., diverged long ago in evolution. In insects, the brain contains numerous different types of neurosecretory cells. From these cells axons project into the retrocerebral complex, which normally consists of a pair of CC and a pair of CA (Fig. 1b). In some insect species the CC are fused (the situation in locusts; Fig. 2) and in others, there is also a single CA, like that in flies.

This review focuses specifically on the peptidergic control of the secretory activity of the CC–CA complex of locusts, because this is by far the best

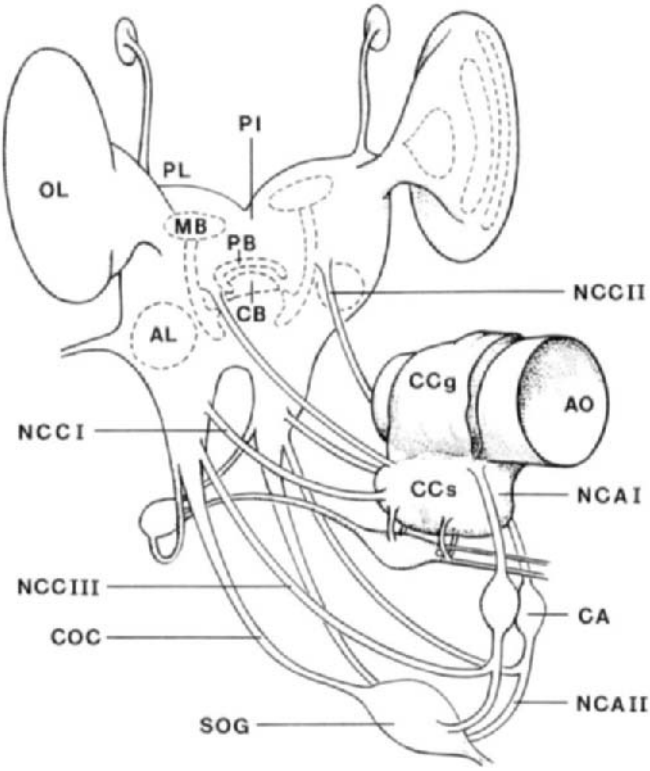

FIG. 2 Diagram of the corpus cardiacum and the corpora allata of *Locusta migratoria* with their connections to the brain via the nervi corporis cardiaci I, II, and III and the nervus corporis allati I, with their connections to the suboesophageal ganglion via the nervus corporis allati II. AO, aorta; AL, antennal lobe; CA, corpus allatum; CB, central body; CCg, glandular lobe of the corpus cardiacum; CCs, storage lobe of the corpus cardiacum; COC, circumoesophageal connectives; MB, mushroom body; NCA I, nervus corporis allati I; NCA II, nervus corporis allati II; NCC I, nervus corporis cardiaci I; NCC II, nervus corporis cadiaci II; NCC III, nervus corporis cardiaci III; OL, optic lobe; PB, protocerebral bridge; PI, pars intercerebralis; PL, pars lateralis; SOG, suboesophageal ganglion.

documented insect model system. The CC and the CA are neuroendocrine organs and the major sites of synthesis and release of adipokinetic hormones (AKHs) (Mayer and Candy, 1969) and juvenile hormone (JH) (Pratt and Tobe, 1974), respectively.

The CC contains a storage lobe (CCs) fused to the ventral wall of the aorta, and two glandular lobes (CCg) fused with the lateral walls of the aorta. In the storage lobe of the CC, neurosecretory products from neurosecretory cells in the brain are stored and released. Therefore, the storage part is the neurohemal part of the CC. Functionally, it corresponds to the

anterior pituitary of vertebrates. The glandular part contains glandular cells synthesizing adipokinetic hormones and two 6-kDa dimeric peptides (Schooneveld *et al.*, 1983; Hietter *et al.*, 1989; Noyes and Schaffer, 1990). This part corresponds to the posterior part of the pituitary. During flight of *Locusta migratoria* the adipokinetic hormones Lom-AKH-1, Lom-AKH-2, and Lom-AKH-3 are released from the CC (Mayer and Candy, 1969; Stone *et al.*, 1976; Carlsen *et al.*, 1979; Orchard and Lange, 1983a; Oudejans *et al.*, 1991). These hormones induce the mobilization of lipids from the fat body (Orchard and Lange, 1983b) and induce the synthesis of flight-specific lipophorins (Van der Horst *et al.*, 1979). Lom-AKH-1 is also an activator of glycogen phosphorylase in the fat body of the locust (Gäde, 1981). In the migratory locust, signal transduction of AKH in the fat body involves the formation of cAMP. The AKH receptor(s) is coupled to cAMP formation and glycogen phosphorylase activation via the stimulatory guanine nucleotide-binding protein (Vroemen *et al.*, 1995a). The elevation of fat body cAMP levels and the activation of phosphorylase by the AKHs *in vitro* depend on the presence of extracellular Ca^{2+}, a concentration of 1.5 m*M* being required for maximal activation by AKHs (Van Marrewijk *et al.*, 1993; Vroemen *et al.*, 1995b). From the CC, other peptides have been isolated, one of which influences ileal transport (Audsley *et al.*, 1992a, c; Hétru *et al.*, 1991; Hietter *et al.*, 1990).

The CA are the major sites of synthesis and release of juvenile hormones (Pratt and Tobe, 1974). In locusts, the CA belong to the semicentralized type: they remain distinctive and are connected to the CC by the nervus corporis allati I (Cassier and Fain-Maurel, 1970). During insect development, JH contributes to maintaining the juvenile form. In the adult stage, the same hormone regulates many reproductive functions including vitellogenesis (Steel and Davey, 1985).

II. Activities of the Corpus Cardiacum and the Corpora Allata

A. The Retrocerebral Glandular Complex and Its Connections to the Brain and Suboesophageal Ganglion

In locusts, the CC is connected to the brain via three paired nerves: the nervi corporis cardiaci I, II, and III (NCC I, II, and III) (Fig. 2). Axons of the NCC I originate from the contralateral median neurosecretory cell bodies and from the ipsilateral tritocerebral neurosecretory cell bodies (Rademakers, 1977; Konings *et al.*, 1988b). Anterograde labeling reveals fibers in the CCs but not in the CCg. Consequently, the axons of the NCC I

may only have an indirect effect on the activity of the adipokinetic cells in the CCg but neurohumoral substances released by the NCC I in the CCs might reach the adipokinetic cells (Konings *et al.,* 1989) (Fig. 3). Axons of the NCC II arise from cell bodies of an extralateral group (about 15 cells) and a lateral group (about 30 cells) in the protocerebrum (Konings *et al.,* 1989). These fibers are present in the CCg and form synapses with the adipokinetic hormone cells (Rademakers, 1977; Pow and Golding, 1987). Fibers of the NCC II are also present in the CCs and probably originate from lateral and extralateral neurosecretory cells of the brain (Konings *et al.,* 1989) (Fig. 4). The NCC III is the most conspicuous one. Backfilling of this nerve revealed 3 neurons in the tritocerebral lobe of the brain (Mason, 1973; Aubele and Klemm, 1977; Bräunig, 1990). In the suboesopha-

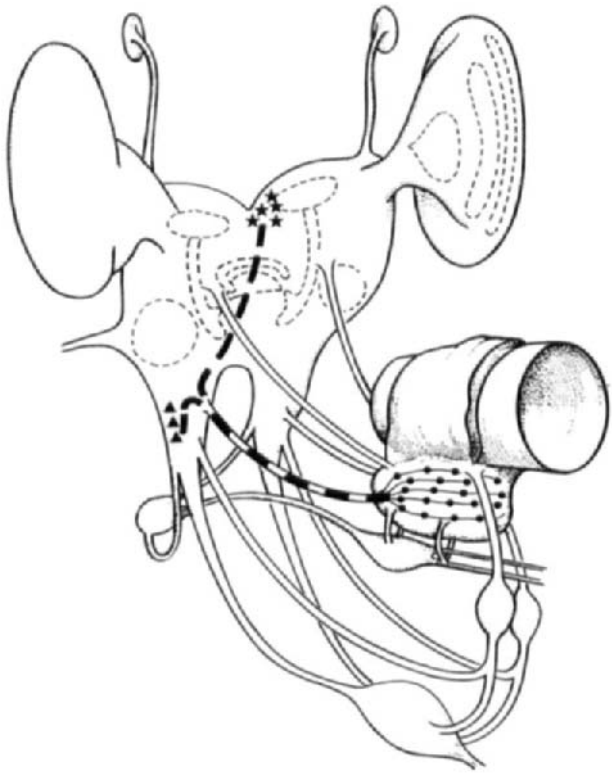

FIG. 3 The origins of the axons of the NCC I, their trajectories, and their projections into the CC. Neurosecretory cells in the pars intercerebralis of the protocerebrum (stars) and in the externolateral part of the tritocerebrum (triangles) and their projections into the CCs.

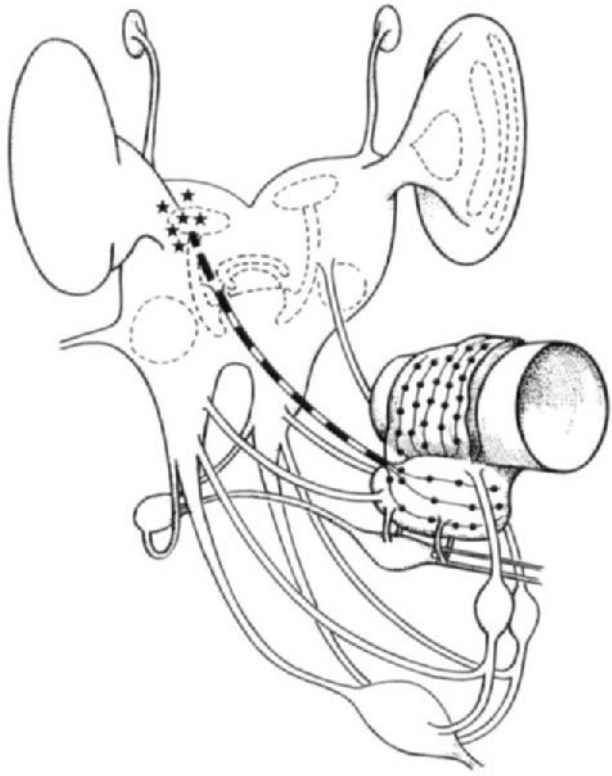

FIG. 4 The origins of the axons of the NCC II, their trajectories, and their projections into the CC. Neurosecretory cells in the pars lateralis of the protocerebrum (stars) and their projections into the CCs and CCg.

geal ganglion (SOG) 3 cell bodies contralateral to the filled nerve, 1 large ventral median cell body, and 6 cell bodies located along the dorsal midline of the ganglion with fibers projecting in the NCC III are found. The NCC III meets the NCA II, passes around the CA, continues toward the CC via the NCA I, and has endings in the CCs (Bräunig, 1990) (Fig. 5).

The CA are connected to the brain and the suboesophageal ganglion via the nervi corporis allati I and II (NCA I, NCA II), respectively. Retrograde filling of the NCA I reveals that cell bodies at the rostroventral side of the calyx of the mushroom body project via the NCC II and through the CCs to the ipsilateral CA (Konings *et al.,* 1989) and 2 cells in each pars lateralis have bilateral projections (Virant-Doberlet *et al.,* 1994). Electrophysiological experiments demonstrated that 13 cells in each pars lateralis innervate

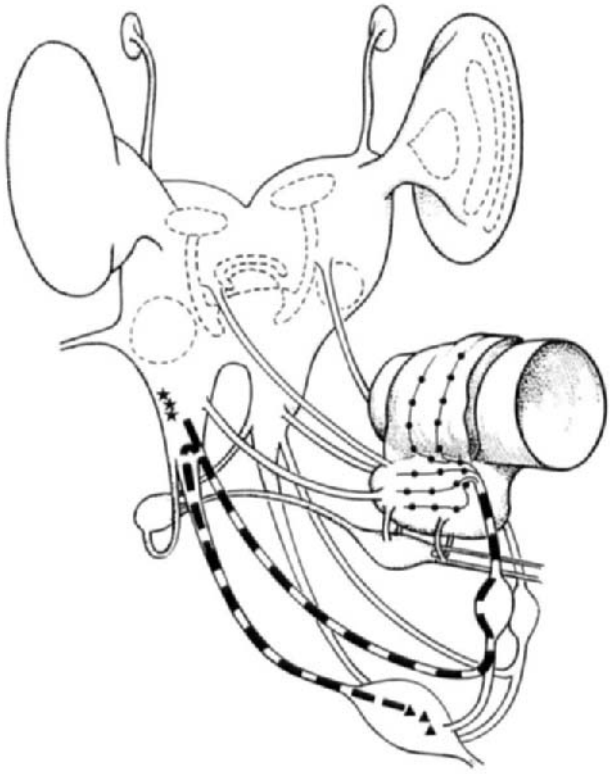

FIG. 5 The origins of the axons of the NCC III, their trajectories, and their projections into the CC. Neurosecretory cells in the suboesophageal ganglion (triangles) and the tritocerebrum (stars) and their projections to the CC. The axons pass the CA.

the ipsilateral CA, while 2 cells in each pars lateralis innervate both glands (Horseman *et al.,* 1994) (Fig. 6). Two populations of cells located ventrally in the suboesophageal ganglion, an anterior group and a posterior one, project into the retrocerebral complex via the NCA II. They project to the CC and arborize in both the storage and the glandular parts of the ipsilateral side. They bypass the CA but form putative release sites on the surface of nerve branches in the vicinity of these glands (Bräunig *et al.,* 1996). Fibers in the NCA II might project further via the NCA I to the CCs and CCg (Fig. 7). Although modulation of JH production via a hormonal route may also occur, there is strong evidence that an important mechanism involves brain neurosecretory cells that project to each CA (Tobe and Stay, 1985).

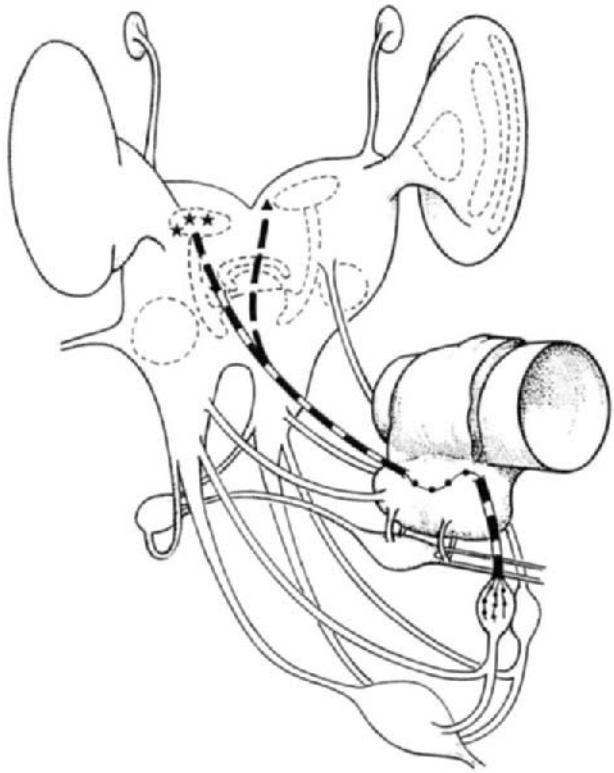

FIG. 6 The origins of the axons of the NCA I, their trajectories, and their projections into the CA. Neurosecretory cells in the rostroventral part of the pars lateralis of the protocerebrum (stars) and their projections to the CA. Two neurosecretory cells projecting to the contralateral CA and to the ipsilateral CA (triangle); the ipsilateral trajectories are not shown.

B. Molecules Controlling the Corpus Cardiacum and Corpora Allata

The CC–CA system in insects is subject to a complex of neural and humoral regulatory mechanisms involving several neural and endocrine messengers, which include neuropeptides as well as small-molecule messengers. Octopamine and cAMP stimulate the release of AKH from the CC (Pannabecker and Orchard, 1986). Recent studies, however, indicate that octopamine, dopamine, tyramine, and serotonin all potentiate the release of adipokinetic hormone induced by cAMP-activating agents. None of them has an effect on its own. Octopamine and tyramine are not detectable in the CC (Konings *et al.,* 1988a). The absence of octopamine in the CC indicates that octopa-

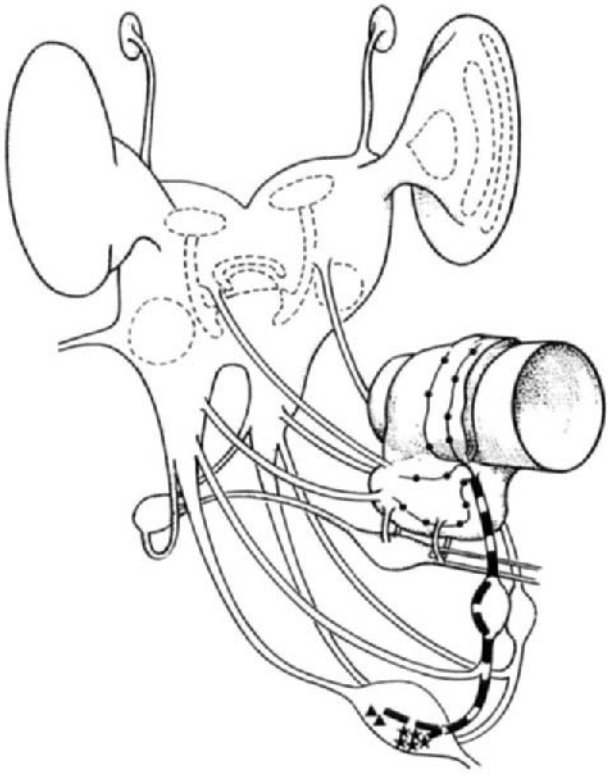

FIG. 7 The origins of the axons of the NCA II, their trajectories, and their projections into the CC. Neurosecretory cells anterior (triangles) and posterior (stars) in the suboesophageal ganglion and their projections to the CCs and CCg.

mine is not the neurotransmitter that initiates release of AKH. At the onset of flight, the octopamine titer in the hemolymph rises rapidly (Goosey and Candy, 1980; Orchard, 1982). It is possible, therefore, that octopamine as a humoral factor from the hemolymph, which bathes the CC, has a modulatory action on an already-initiated AKH release (Passier *et al.*, 1995). On the other hand, dopamine and serotonin are present in the neurohemal part of the CC (Lafon-Cazal and Arluison, 1976; David and Lafon-Cazal, 1979; Orchard *et al.*, 1986; Passier, 1996) and may therefore serve as regulatory substances that play a modulatory role in the release of AKH.

Locustatachykinin (Lom-TK-1) is able to induce Lom-AKH-1 release in a dose-dependent manner, although at high concentrations (ED_{50}: $\pm 50\ \mu M$) (Nässel *et al.*, 1995). The FMRF-amide related peptides inhibit *in vitro* the IBMX-induced AKH release (Passier, 1996). Crustacean cardio-

active peptide induces the release of AKH in a dose-dependent manner in concentrations ranging from 10^{-5} to 10^{-9} *M* (Veelaert *et al.*, 1997).

Since 1989, a number of neuropeptides that either stimulate or inhibit JH production by the CA have been isolated from brains of a few insect species (Table I). These peptides are termed allatotropins and allatostatins, respectively. Four families have been reported: (1) The allatostatin A family or the *Diploptera punctata* allatostatin family isolated and identified from *D. punctata* (Pratt *et al.*, 1989, 1991; Donly *et al.*, 1993; Woodhead *et al.*, 1989, 1994), *Periplaneta americana* (Weaver *et al.*, 1994; Ding *et al.*, 1996), *Blatella germanica* (Bellés *et al.*, 1994), *Gryllus bimaculatus* (Lorenz *et al.*, 1995b), and *Calliphora vomitoria* (Duve *et al.*, 1993). (2) The allatostatin B family or the *G. bimaculatus* allatostatins family isolated from *G. bimaculatus* (Lorenz *et al.*, 1995a). Myoinhibiting peptides belonging to this family have been isolated from *L. migratoria* (Schoofs *et al.*, 1991b) and *Manduca sexta* (Blackburn *et al.*, 1995). (3) The allatostatin C family or the *M. sexta* allatostatin family isolated from *M. sexta* (Kramer *et al.*, 1991). (4) The *M. sexta* allatotropin family (Kataoka *et al.*, 1989; Paemen *et al.*, 1991). The callatostatins isolated from *C. vomitoria* are inhibitors of JH biosynthesis by the CA of *D. punctata* but are inactive in the fly itself (Duve *et al.*, 1993).

In adult locusts, juvenile hormone is required for normal development, as shown by experiments in which the CA were surgically removed or destroyed by precocene treatment (Couillaud *et al.*, 1984). When animals lacking CA are treated with synthetic juvenile hormone, reproduction becomes normal again. Such experiments clearly show that the CA have to be active, not necessarily continuously, during the adult stage. However, problems arise when one tries to measure juvenile hormone biosynthesis by the CA under *in vitro* conditions. In the cockroach *D. punctata,* a very reliable assay system has been elaborated by Tobe and Clark (1985a,b) and the CA are quite active. Addition of compounds with inhibitory activity reduces the rate of juvenile hormone biosynthesis. This assay system has been used to isolate and characterize the allatostatins of the cockroaches and the cricket. Especially in locusts, JH biosynthesis is very variable and the basal rate is very low. Even between two corpora allata of the same pair, there can be a difference in the rate of JH biosynthesis of about 1000 times. When this bioassay system is applied to *Schistocerca gregaria,* highly variable results are also obtained. Hardly any JH biosynthesis activity can be found this way. Although there is evidence that neurosecretory cells are involved in the control of JH biosynthesis in locusts, the nature of the factors involved has as yet not been determined. Neurosecretory cells of the pars lateralis have been suggested to contain a strong allatostimulating factor (Couillaud and Girardie, 1985). Either sectioning the NCC II or NCA I or destroying the lateral neurosecretory cells results in a decrease in JH biosynthesis by the CA (Girardie *et al.*, 1981; Couillaud and Girardie,

TABLE I

Allatostatins, Allatotropins, and Related Peptides Isolated from Different Insect Species

Allatostatin A family		
Diploptera punctata		
Dip-AST-1	**LYDFGLa**	Donly *et al.* (1993)
Dip-AST-2	AYSYVSEYKRLPVYN**FGLa**	Pratt *et al.* (1991); Donly *et al.* (1993)
Dip-AST-3	SKMYG**FGLa**	Donly *et al.* (1993)
Dip-AST-4	DGRMYS**FGLa**	Donly *et al.* (1993); Woodhead *et al.* (1994)
Dip-AST-5	DRLYS**FGLa**	Woodhead *et al.* (1989); Donly *et al.* (1993)
Dip-AST-6	ARPYS**FGLa**	Donly *et al.* (1993)
Dip-AST-7	APSGAQRLYG**FGLa**	Woodhead *et al.* (1989); Pratt *et al.* (1989); Donly *et al.* (1993)
Dip-AST-8	GGSLYS**FGLa**	Woodhead *et al.* (1989); Donly *et al.* (1993)
Dip-AST-9	GDGRLYA**FGLa**	Woodhead *et al.* (1989); Donly *et al.* (1993)
Dip-AST-10	PVNSGRSSGSRFN**FGLa**	Donly *et al.* (1993)
Dip-AST-11	YPQEHRFS**FGLa**	Woodhead *et al.* (1994); Donly *et al.* (1993)
Dip-AST-12	PFN**FGLa**	Donly *et al.* (1993)
Dip-AST-13	IPMYD**FGIa**	Donly *et al.* (1993)
Blatella germanica		
Blg-AST-1	**LYDFGLa**	Bellés *et al.* (1994)
Blg-AST-2	DRLYS**FGLa**	Bellés *et al.* (1994)
Blg-AST-3	AGSDGRLYS**FGLa**	Bellés *et al.* (1994)
Blg-AST-4	APSSAQRLYG**FGLa**	Bellés *et al.* (1994)
Periplaneta Americana		
Pea-AST-1	**LYDFGLa**	Ding *et al.* (1996)
Pea-AST-2	AYSYVSEYKRLPVYN**FGLa**	Ding *et al.* (1996)
Pea-AST-3	SKMYG**FGLa**	Ding *et al.* (1996)
Pea-AST-4	SGNDGRLYS**FGLa**	Ding *et al.* (1996)
Pea-AST-5	DRMYS**FGLa**	Ding *et al.* (1996)
Pea-AST-6	ARPYS**FGLa**	Ding *et al.* (1996)
Pea-AST-7	SPSGMQRLYG**FGLa**	Weaver *et al.* (1994); Ding *et al.* (1996)
Pea-AST-8	GGSMYS**FGLa**	Ding *et al.* (1996)
Pea-AST-9	ADGRLYA**FGLa**	Weaver *et al.* (1994); Ding *et al.* (1996)

Pea-AST-10	PVSSARQTGSRFN**FGLa**	Ding *et al.* (1996)
Pea-AST-11	SPQGHRFS**FGLa**	Ding *et al.* (1996)
Pea-AST-12	SLH**YAFGLa**	Ding *et al.* (1996)
Pea-AST-13	**PYNFGLa**	Ding *et al.* (1996)
Pea-AST-14	IPM**YDFGIa**	Ding *et al.* (1996)
Gryllus bimaculatus		
Grb-AST-1A	AQHQ**YSFGLa**	Lorenz *et al.* (1995b)
Grb-AST-2A	AGGRQ**YSFGLa**	Lorenz *et al.* (1995b)
Schistocerca gregaria		
Scg-AST-1	LCD**FGVa**	Vanden Broeck *et al.* (1996)
Scg-AST-2	AYTYVSEYKRLPV**YNFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-2$^{11-18}$	LPV**YNFGLa**	Veelaert *et al.* (1996b); Vanden Broeck *et al.* (1996)
Scg-AST-3	ATGAASL**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-4	GPRT**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-5	GRL**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-6	ARP**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-7	AGPAPSRL**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-8	EGRM**YSFGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Scg-AST-9	PLYGGDRRFS**FGLa**	Vanden Broeck *et al.* (1996)
Scg-AST-10	APAEHRFS**FGLa**	Veelaert *et al.* (1996a); Vanden Broeck *et al.* (1996)
Calliphora vomitoria		
Leu-callatostatin-1	DPLNEERRANR**YGFGLa**	Duve *et al.* (1993)
Leu-callatostatin-2	LNEERRANR**YGFGLa**	Duve *et al.* (1993)
Leu-callatostatin-3	ANR**YGFGLa**	Duve *et al.* (1993)
Leu-callatostatin-4	NRP**YSFGLa**	Duve *et al.* (1993)
Met-callatostatin	GPP**YDFGMa**	Duve *et al.* (1993)
[Hyp^3]Met-callatostatin	GP[Hyp]**YDFGMa**	Duve *et al.* (1994)
[Hyp^2]Met-callatostatin	G[Hyp]P**YDFGMa**	Duve *et al.* (1995a)
des Gly-Pro Met-callatostatin	P**YDFGMa**	Duve *et al.* (1995b)

(*continues*)

TABLE I (*Continued*)

Allatostatin B family		
Gryllus bimaculatus		
Grb-AST-1B	**QWQDLNGGWa**	Lorenz *et al.* (1995a)
Grb-AST-2B	**GWRDLNGGWa**	Lorenz *et al.* (1995a)
Grb-AST-3B	**AWRDLSGGWa**	Lorenz *et al.* (1995a)
Grb-AST-4B	**AWERFHGWSa**	Lorenz *et al.* (1995a)
Manduca sexta		
Mas-MIP-1	**AWQDLNSAWa**	Blackburn *et al.* (1995)
Mas-MIP-2	**GWQDLNSAWa**	Blackburn *et al.* (1995)
Locusta migratoria		
Lom-MIP	**AWQDLNAGWa**	Schoofs *et al.* (1991b)
Allatostatin C family		
Manduca sexta		
Mas-AST	pQVRFRQCYFNPISCF	Kramer *et al.* (1991)
Allatotropin family		
Manduca sexta		
Mas-Atr	**GFKNVEMMTARGFa**	Kataoka *et al.* (1989)
Locusta migratoria		
Lom-AG-MT-1	**GFKNVALSTARGFa**	Paemen *et al.* (1991)

Note. [Hyp], hydroxyproline; boldface letters represent consensus sequence; a, amidation.

1985; Tobe *et al.,* 1977). Brain extracts of *L. migratoria* display allatotropic activity (Ferenz and Diehl 1983; Granger *et al.,* 1984; Ferenz, 1984; Gadot and Applebaum, 1985; Rembold *et al.,* 1986; Couillaud and Girardie, 1990; Unni *et al.,* 1991; Lehmberg *et al.,* 1992; Veelaert *et al.,* 1995b; Veelaert, 1996), but the nature of these stimulatory factors remains unknown.

Recently, schistostatins have been isolated from the brain of *S. gregaria.* These peptides belong to the allatostatin A family but *in vitro* they are not inhibitors of juvenile hormone biosynthesis in the locust (Veelaert *et al.,* 1996a; Vanden Broeck *et al.,* 1996).

III. Locust Endogenous Neuropeptides

Adipokinetic hormone was the first neuropeptide isolated from locusts (Stone *et al.,* 1976). Since then, about 40 peptides have been isolated from locusts and this list continues to grow (Schoofs *et al.,* 1997a). Table II shows the sequences of all peptides isolated from locusts that possibly cross-react with antisera used for immunocytochemical staining in the central nervous system of locusts. Table III shows which part of the retrocerebral glandular complex contains peptide-like immunoreactivity.

A. Neuropeptides Present in the Corpus Cardiacum–Corpora Allata Complex

1. Accessory Gland Myotropin

Locusta accessory gland myotropin I (Lom-AG-MT-1) is the first peptide isolated from insect male accessory glands. It stimulates the contractility of the oviduct in locusts (Paemen *et al.,* 1991). Both the male accessory glands and the central nervous system contain Lom-AG-MT-like immunoreactivity. Immunoreactive cell bodies are found in the lateral protocerebrum, the lateral part of the medulla of the optic lobe, and the dorsolateral protocerebrum. One reactive neuron is observed in the dorsal protocerebrum with an axon leading down into the neuropil. Axons in the storage part of the CC stain with the Lom-AG-MT antiserum, but no immunoreactivity is found in the CCg (Paemen *et al.,* 1992). Although this peptide has similarities with the allatotropin of *M. sexta,* the CA are immunonegative. Lom-AG-MT-2 has also been isolated from locust male accessory glands and stimulates contractility of the oviduct in locusts (Paemen, 1991). The presence of Lom-AG-MT-2-like material in the retrocerebral complex has not been investigated.

TABLE II

Sequences of Endogenous Neuropeptides of Which Immunoreactive-like Material Was Demonstrated in Locusts

Accessory gland myotropin		
Lom-AG-MT-1	GPKNVALSTARGFa	Paemen *et al.* (1991)
Lom-AG-MT-2	AHRFAAEDFGALDFGALDTA	Paemen (1991)
Adipokinetic hormone		
Lom-AKH-1	**pELNF**TPNWGTa	Stone *et al.* (1976)
Lom-AKH-1^{4-10}	**F**TPNWGTa	Schoofs *et al.* (1993b)
Lom-AKH-2	**pELNF**SAGWa	Carlsen *et al.* (1979)
Lom-AKH-3	**pELNF**TPWWa	Oudejans *et al.* (1991)
Scg-AKH-1	**pELNF**TPNWGTa	Stone *et al.* (1976)
Scg-AKH-2	**pELNF**STGWa	Siegert *et al.* (1985)
Phl-AKH-1	**pELNF**STGWa	Gäde and Kellner (1995)
Phl-AKH-2	**pELTF**TPNWGSa	Gäde and Kellner (1995)
Arginine vasopressin-like insect diuretic hormone		
Lom-F1	CLITNCPRG-NH_2	Proux *et al.* (1987)
Lom-F2	CLITNCPRG-NH_2 CLITNCPRG-NH_2	Proux *et al.* (1987)
Corazonin		
Sca-COR	pETFQYSRGWTNa	Veenstra (1991)
Crustacean cardioactive peptide		
Lom-CCAP	PFCNAFTGCa	Stangier *et al.* (1989)
Scg-CCAP	PFCNAFTGCa	Veelaert *et al.* (1997)
Diuretic peptide		
Lom-DP	MGMGPSLSIVNPMDVLRQRLLLEIARRRL RDAEEQIKANKDFLQQIa	Lehmberg *et al.* (1991)

FLRFamide		
Scg-FLRFamide (SchistoFLRFamide)	**PDVDHVFLRFa**	Robb *et al.* (1989)
Lom-FLRFamide-1	**PDVDHVFLRFa**	Schoofs *et al.* (1993a)
Lom-FLRFamide-2	**ADVGHVFLRFa**	Peeff *et al.* (1994)
Insulin-related molecule		
A chain	GVFDECCRKSCSISELQTYCG	Hétru *et al.* (1991)
B chain	SGAPQPVARYGEKLSNALKLVCRGGNYNTMF	Hétru *et al.* (1991)
C chain	ASQDVSDSESEDNYYSGQSADEAAEAAAAA LPPYPILARPSAGGLLTGAV	Hietter *et al.* (1989)
Ion transport peptide	SFFDIQCKGVYDKSSIFARLDRICEDCYNLF REPPQLHSLCRSDCFKSPYFKGCLQALLLID EEEKFNQMMVEILG	Meredith *et al.* (1996)
Kinin		
Lom-KIN	AFSSWGa	Schoofs *et al.* (1992b)
Myoinhibin		
Lom-MIH	pEXYXKQSAFNAVSa	Schoofs *et al.* (1994)
Myotropin		
Lom-MT-1	GAVPAAQ**FSPRLa**	Schoofs *et al.* (1990d)
Lom-MT-2	EGD**FTPRLa**	Schoofs *et al.* (1990b)
Lom-MT-3	RQQP**FVPRLa**	Schoofs *et al.* (1992a)
Lom-MT-4	RLHQNGMP**FSPRLa**	Schoofs *et al.* (1992a)
Neuroparsin		
Lom-Neuroparsin-B	NPISRSCEGANCVVDLTRCEYGDVTDFFGR KVCKGPGDKCGGPYELHGKCGVMDCRCGLC SGCSLHLQCFFFEGGLPSSC	Girardie *et al.* (1989)
Lom-Neuroparsin-A-1	NPIS**R-Neuroparsin-B**	Girardie *et al.* (1990)

(*continues*)

TABLE II (*Continued*)

Lom-Neuroparsin-A-2	**ISR-Neuroparsin-B**	Girardie *et al.* (1990)
Lom-Neuroparsin-A-3	**SR-Neuroparsin-B**	Girardie *et al.* (1990)
Lom-Neuroparsin-A-4	**R-Neuroparsin-B**	Girardie *et al.* (1990)
Ovary maturating parsin		
Lom-OMP	YYEAPPDGRHLLLQPAPAAPAVPA (A or S) PASWPHQQRRQALDEFAAAAA AAADAQFQDEEEDGGRRV	Girardie *et al.* (1991)
Proctolin		
Lom-proctolin	RYLPT	Schoofs *et al.* (1993c)
Pyrokinin		
Lom-PK-1	pEDSGDEWPQQP**FVPRLa**	Schoofs *et al.* (1991a)
Lom-PK-2	pESVPT**FTPRLa**	Schoofs *et al.* (1993c)
Sulfakinin		
Lom-SK	pELASDDY (SO_3H) GHMRFa	Schoofs *et al.* (1990e)
Tachykinin		
Lom-TK-1	GPS**GFYGVRa**	Schoofs *et al.* (1990c)
Lom-TK-2	APLS**GFYGVRa**	Schoofs *et al.* (1990c)
Lom-TK-3	APQA**GFYGVRa**	Schoofs *et al.* (1990a)
Lom-TK-4	APSL**GFHGVRa**	Schoofs *et al.* (1990a)
Lom-TK-5	XPSW**FYGVRa**	Schoofs *et al.* (1993c)

Note. X, unknown amino acid; boldface letters represent consensus sequence; a, amidation.

TABLE III

Endogenous Neuropeptides of Which Immunoreactive-like Material Was Demonstrated in the Corpus Allatum (CA), the Storage Part of the Corpus Cardiacum (CCs), and the Glandular Part of the Corpus Cardiacum (CCg)

	CA	CCs	CCg	Reference
AVP-like IDH-like	−	−	−	Rémy and Girardie (1980)
Lom-AG-MT-1-like	−	+	−	Paemen *et al.* (1992)
Lom-AG-MT-2-like	−	−	−	Paemen (1991)
Lom-AKH-like	?	+	+	Schooneveld *et al.* (1983)
Lom-CCAP-like	?	+	+	Dircksen and Homberg (1995)
Lom-DP-like	+	+	−	Patel *et al.* (1994)
Lom-KIN-like	?	?	?	Nässel (1993b)
Lom-MIH-like	−	−	−	Schoofs *et al.* (1994)
Lom-MIP-like	+	+	+	Schoofs *et al.* (1996)
Lom-MT-like	−	+	−	Schoofs *et al.* (1992c)
Lom-OMP-like	+	+	−	Richard and Girardie (1992)
Lom-TK-like	−	+	+	Nässel *et al.* (1995)
Neuroparsin-like	?	+	−	Bourême *et al.* (1987)
Scg-AST-like	+	+	−	Veelaert *et al.* (1995a)
Scg-FLRFamide-like	+	+	+	Myers and Evans (1987)

Note. +, contains immunoreactive material; −, contains no immunoreactive material; ?, not reported.

2. Corpus Cardiacum Intrinsic Peptides: AKHs and 6-kDa Peptides

Two adipokinetic hormones, Scg-AKH-1 (Lom-AKH-1) and Scg-AKH-2 (Lom-AKH-2), have been isolated from both locusts, *S. gregaria* and *L. migratoria* (Stone *et al.*, 1976), but Lom-AKH-3 has been isolated from *L. migratoria* only (Oudejans *et al.*, 1991). A degradation product of Lom-AKH-1:Lom-AKH-1^{4-10} has also been isolated from *L. migratoria* (Schoofs *et al.*, 1993b). The cDNAs encoding the preproadipokinetic hormones 1, 2, and 3, respectively, of *L. migratoria* have been isolated and sequenced. *In situ* hybridization shows that the three different Lom-AKH-1, 2, and 3 precursors are colocalized in cell bodies of the CCg (Bogerd *et al.*, 1995). Flight activity increases steady-state levels of AKH mRNAs in the CC. AKH-like immunoreactivity is found in the glandular cells of the CCg but also in neurosecretory cell bodies of the brain. Cells of CCg are revealed by both an N-terminal and a C-terminal specific antiserum. On the other hand, neurons in the central nervous system are only revealed by the C-

terminal specific antiserum (Schooneveld *et al.*, 1986). NCC I contains immunoreactive fibers projecting to the CCs (Schooneveld *et al.*, 1983, 1985). This indicates that a C-terminal AKH-like peptide might be involved in controlling the release of AKH. Immunoreactivity is found neither in CA nor in NCA I and II.

During purification of the major peaks of a locust CC extract, two 6-kDa peptides have been identified (Hietter *et al.*, 1989). Their function is unknown. Immunocytochemical studies show that the 6-kDa peptides are contained in the secretory granules of the intrinsic cells of the glandular lobes of the CC.

3. Corazonin

Corazonin is a cardioactive peptide that stimulates the heart rhythm. It was first isolated from the cockroach *P. americana.* In this species it is synthesized in a small group of lateral neurosecretory cells in the brain whose axons end in the ipsilateral part of the CC (Veenstra, 1989). Corazonin (Sca-COR) has been isolated from *Schistocerca americana* by screening HPLC fractions with an ELISA (Veenstra, 1991). The immunodistribution of corazonin in the locust has not been investigated.

4. Crustacean Cardioactive Peptide (CCAP)

By screening chromatographically purified fractions in a bioassay to measure adipokinetic hormone release, a peptidergic adipokinetic hormone releasing factor (Scg-CCAP) has been isolated form 7000 brains of the dessert locust *S. gregaria.* Scg-CCAP stimulates the release of adipokinetic hormone in a dose-dependent manner in *S. gregaria* (Veelaert *et al.*, 1997). This is the first demonstration in invertebrates of a peptide with a function analogous to that of the hypothalamic releasing factors of vertebrates. The peptide has first been isolated from the shore crab *Carcinus maenas* as a cardioactive peptide, CCAP (Stangier *et al.*, 1987). Later, CCAP was isolated from *L. migratoria* by affinity chromatography (Stangier *et al.*, 1989).

Four neurons of the first SOG neuromere, most likely corresponding to the anterior NCA II cells, contain CCAP-like immunoreactive material. Their axons join and project through the connectives and the tritocerebrum into the NCC III. The immunoreactive fibers pass the CA and arborize in the CCs and rarely in the CCg (Dircksen *et al.*, 1991; Dircksen and Homberg, 1995). In the CC, CCAP might be released into the hemolymph (Dircksen and Homberg, 1995). Indeed, CCAP meets the requirements for hormonal status in that it is active at low concentrations. Since in insects other functions are described for CCAP, it might act as a pleiotropic hormone. During flight, it activates the heartbeat, resulting in increased blood circulation

(Tublitz, 1989). In the meantime, it might facilitate the release of adipokinetic hormone, which stimulates the mobilization of lipids and carbohydrates from the fat body. These serve as energy substrates for flight muscle activity.

No Lom-CCAP-immunoreactive cell bodies with fibers projecting to the retrocerebral complex are found in the brain. Lom-CCAP is a potent myostimulator of the hindgut of *L. migratoria* with a significant effect being observable at concentrations of 10^{-10} *M* (Stangier *et al.*, 1989).

5. Diuretic Peptide

Locusta diuretic peptide (Lom-DP) is a potent stimulant of fluid secretion and cyclic AMP production by locust Malpighian tubules (Lehmberg *et al.*, 1991; Kay *et al.*, 1991). Many strongly immunostained neurons are found in the pars intercerebralis with axons projecting into the NCC I. The staining of the CCs is most dense in the periphery of the lobe, which faces the hemocoel. The glandular lobe has very few immunoreactive endings (Patel *et al.*, 1994). From the CC, Lom-DP is released into the hemolymph, where it has been identified by mass spectrometry (Patel *et al.*, 1995) and by radioimmunoassay (Audsley *et al.*, 1997).

6. FLRFamide Peptide Family

SchistoFLRFamide (Scg-FLRFamide-1) has been isolated from brains of *S. gregaria* by monitoring HPLC fractions by radioimmunoassay (Robb *et al.*, 1989). SchistoFLRFamide (Lom-FLRFamide-1) has been isolated from brains of *L. migratoria* based on its ability to suppress spontaneous contractions of the hindgut of *Leucophaea maderae* (Schoofs *et al.*, 1993a). Recently, ADVGHVFLRFamide (Lom-FLRFamide-2) has been isolated from the central nervous system of *L. migratoria* (Peeff *et al.*, 1994). The peptides have a potent inhibitory action on heart rhythm (Robb and Evans, 1990) and inhibit or reduce spontaneous contractions of the oviduct of *L. migratoria* (Schoofs *et al.*, 1993a). FMRFamide potentiates twitch tension in the extensor tibiae muscle of *S. gregaria* (Walther and Schiebe, 1987) and mimics the effect of serotonin and dopamine in the salivary nerve (Baines and Tyrer, 1989).

Six lateral protocerebral neurosecretory cells, containing FMRFamide-like immunoreactivity, project into the NCC II and reach the CCs (Rémy *et al.*, 1988; Schoofs *et al.*, 1993a). FMRFamide-like immunoreactivity is also present in many cells of the pars intercerebralis and project into the NCC I (Myers and Evans, 1987). FMRFamide-like staining activity changes dramatically during the oviposition cycle in mature adult females. The median neurosecretory cells stain lightly immediately after oviposition and

remain pale until the third day, when staining increases (Sevala *et al.*, 1993). Synaptic contacts on the adipokinetic cells in the CCg also contain FMRFamide-like material (Passier *et al.*, 1994). A single CC of *S. gregaria* contains about 80 fmol of FMRFamide-like peptides, as has been determined by RIA (Robb and Evans, 1990). Lom-FLRFamide-1 seems to be an inhibitor of Lom-AKH-1 release by the CC (Passier *et al.*, 1994; Passier, 1996).

Locustasulfakinin (Lom-SK) (Schoofs *et al.*, 1990e), with a HMRFamide carboxy terminus, probably cross-reacts with the antisera used to demonstrate FLRFamide.

7. FXPRLamide Peptide Family

Six neuropeptides, locustamyotropins 1–4 (Lom-MT) and locustapyrokinins 1–2 (Lom-PK), isolated from *L. migratoria,* share the C-terminal pentapeptide sequence FXPRL-NH_2 (Schoofs *et al.*, 1990b,d, 1991a, 1992a, 1993c). They stimulate contractions of the hindgut of *L. maderae* and of the oviduct of *L. migratoria.* Lom-MT-like immunoreactivity is present in cell bodies of the lateral tritocerebrum that project via the NCC I to the CC. While no labeling is present in the CCg, the CCs facing the dorsal blood vessel contains strong immunoreactivity. In the suboesophageal ganglion immunoreactive cell bodies project into the NCA II and via the NCA I to the CC. The intrinsic cells of the CA are immunonegative (Schoofs *et al.*, 1992c). This is in accordance with the results of Bräunig *et al.* (1996).

8. Insulin-Related Peptide

Locusta insulin-related peptide (Lom-IRP) has been purified from an extract of 500 CC (Hétru *et al.*, 1991). *Locusta* insulin is immunolocalized in neurosecretory cells of the pars intercerebralis–corpora cardiaca system (Goltzené *et al.*, 1992). These cells produce large amounts of a 145-residue precursor that is posttranslationally processed into a 21-residue A chain, a 31-residue B chain, and a C peptide (Lageux *et al.*, 1990). In both the A and the B chains, the cysteine residues are located in positions similar to those in other insulins. The possible function(s) of *L. migratoria* insulin remains to be identified.

9. Myoinhibiting Peptide

Locusta myoinhibiting peptide (Lom-MIP) has been isolated from 9000 brain–CC–CA–suboesophageal ganglion complexes (Schoofs *et al.*, 1991b). Lom-MIP suppresses spontaneous contractions of both the hindgut and the oviduct of *L. migratoria.* Lom-MIP shows sequence similarities with

allatostatins of the B family isolated from the cricket *G. bimaculatus* (Lorenz *et al.*, 1995a). Using a polyclonal antiserum, approximately 12 Lom-MIP immunopositive neurons stain in the brain. The glandular cells in the CC, known to produce adipokinetic hormones, are contacted by Lom-MIP-like immunopositive fibers. The nerve connecting the CC to the CA, the NCA I, also contains immunopositive fibers (Fig. 8) (Schoofs *et al.*, 1996).

10. Neuroparsin

Neuroparsins are disulfide bridge-containing proteins (Girardie *et al.*, 1989, 1990). Neuroparsin A is synthesized in the median region of the brain and transported via the NCC I to the CC. Neuroparsin B is not formed in the brain but only occurs in the CC and has been proposed to be a modified

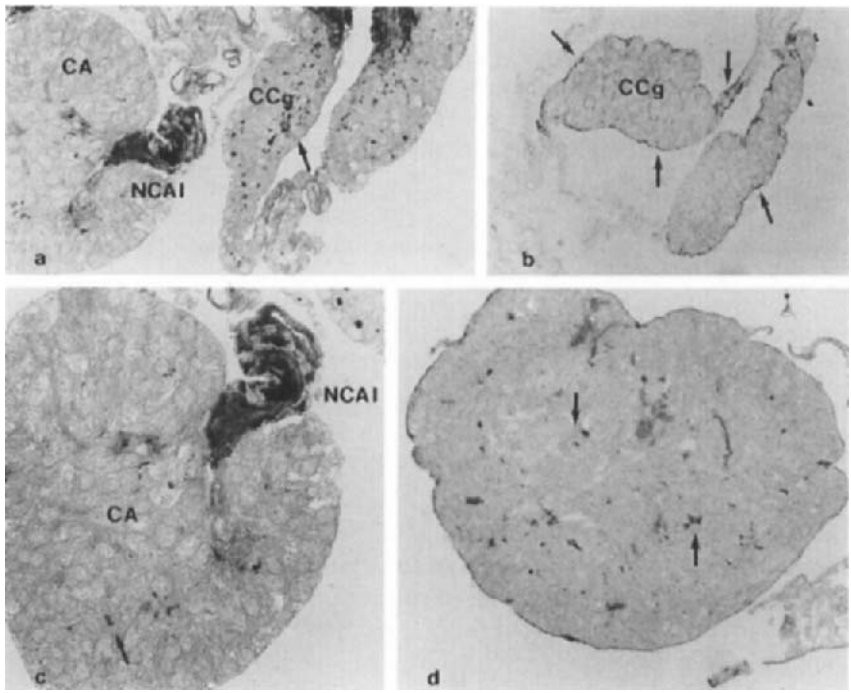

FIG. 8 Locusta myoinhibiting peptide immunoreactive fibers in the corpus cardiacum (CC) and in the corpora allata (CA). (a) Immunoreactive processes in nervus corporis cardiaci II (NCC II) and immunoreactive endings (arrows) in the glandular part (CCg). Original magnification, × 800. Immunoreactive fibers in nervus corporis allati I (NCA I) and endings in corpus allatum (CA). (b) Glandular part of CC (CCg) of another locust. Immunoreactive fibers are only at the periphery. Original magnification, ×800. (c,d) Corpora allata with NCA I and immunoreactive endings (arrows). Original magnification, ×800.

neuroparsin A (Girardie *et al.,* 1986). No immunoreactivity is present in the CCg but the CCs contained strong immunoreactivity (Bourême *et al.,* 1987). Neuroparsins are multifunctional neurohormones: they antagonize the effect of juvenile hormone (Girardie *et al.,* 1987), they stimulate fluid reabsorption of isolated recta (Fournier and Girardie, 1988), and they induce an increase in hemolymph lipid and trehalose levels (Moreau *et al.,* 1988).

11. Ovary Maturating Parsin

Locusta ovary maturating parsin (Lom-OMP) has been purified from neurosecretory lobes of locust CC and is the first gonadotropic hormone sequenced in insects (Girardie *et al.,* 1991). It stimulates ecdyson biosynthesis by the ovary (Girardie and Girardie, 1996). About 250 perikarya are immunoreactive in the median neurosecretory center. These ovary maturating parsin cells have larger and more numerous vesicles than the neuroparsin cells (Tamarelle and Girardie, 1994). The largest group of immunoreactive fibers is that of the two axonal pathways originating from the median neurosecretory perikarya and constituting the NCC I that terminates in the periphery of the CCs. Very few fibers enter via the NCC II. Fibers are rarely seen in the NCA I and the CA also does not stain (Richard and Girardie, 1992). Electron microscopy reveals that Lom-OMP axon terminals occupy a peripheral position close to the aorta wall (Tamarelle and Girardie, 1994).

12. Schistostatins

Nine schistostatins have been chromatographically purified from a brain extract of 7000 desert locusts, *S. gregaria* (Veelaert *et al.,* 1996a,b). They all have the typical Tyr-Xaa-Phe-Gly-Leu-amide C terminus and definitely belong to the cockroach allatostatin A family. They inhibit spontaneous contractions of the lateral oviducts of *S. gregaria* but have no effect on juvenile hormone biosynthesis by the CA of *S. gregaria* (Veelaert *et al.,* 1996a; Devreese *et al.,* 1997). One schistostatin, Scg-AST-2^{11-18}, is a cleavage product of Scg-AST-2 which contains a dibasic cleaving site. While the other isolated schistostatins are inhibitors of juvenile hormone biosynthesis by CA of the cockroach *D. punctata,* Scg-AST-2^{11-18} does not display this effect. It is possible that the presence of Scg-AST-2^{11-18} in *S. gregaria* blocks allatostatin activity or plays a role in negative feedback regulation (Veelaert *et al.,* 1996b). This could explain the failure to demonstrate *in vitro* allatostatic activity by allatostatin-like peptides in the desert locust, the only insect from which this cleavage product has been isolated. The cDNA encoding the precursor polypeptide for schistostatins has been cloned. It

encodes 10 schistostatins, of which 2 have not been chromatographically isolated (Vanden Broeck *et al.,* 1996).

In *S. gregaria,* strongly immunoreactive cells stain in the pars lateralis of the brain with axons extending to and arborizing in the CA and the storage part of the CC (Fig. 9). No immunoreactivity is present in the glandular part of the CC (Veelaert *et al.,* 1995a). In each brain more than 2000 neurons exhibit Dip-AST-1-like immunoreactivity, suggesting a prominent neuroactive role for Dip-AST-1-related peptides in the brain (Vitzthum *et al.,* 1996).

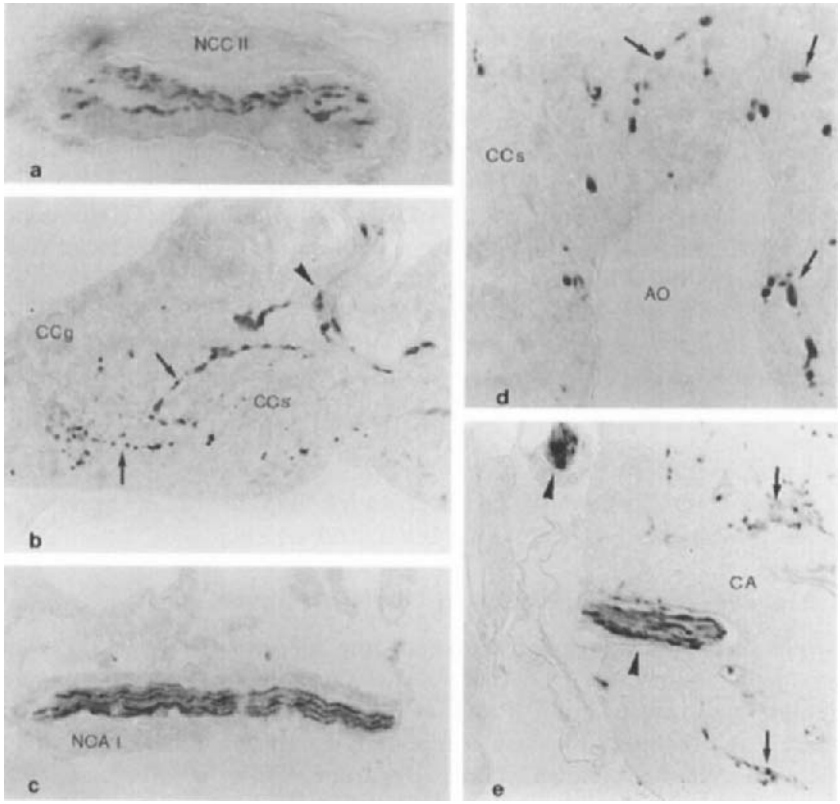

FIG. 9 Schistostatin Immunoreactive fibers leading to the corpus cardiacum (CC) and the corpora allata (CA). (a) Axons in NCC II. Original magnification, ×800. (b) NCC II entering CC (arrowhead) and arborizing in CCs (arrows). Original magnification, ×400. (c) Axons in NCA I. Original magnification, ×800. (d) Immunoreactivity (arrows) in the neurohemal part of the CCs, AO, and aorta. Original magnification, ×800. (e) Immunoreactive fibers of NCA I entering CA and arborizing in CA. Original magnification, ×800.

13. Tachykinins

Locustatachykinins (1–5) (Lom-TK) are isolated from brains of *L. migratoria* (Schoofs *et al.,* 1990a,c, 1993c). They stimulate contractions of the hindgut of *L. maderae.* Lom-TK-like fibers are observed in the NCC II nerves to the CC and a sparse distribution of varicose fibers is detected within the CCg and CCs (Nässel *et al.,* 1995). Electron-microscopical immunocytochemistry reveals that Lom-TK-like axon terminals containing granular vesicles make contact with the glandular cells (Nässel *et al.,* 1995). Lom-TK-1 induces the release of AKH, albeit at high concentrations (ED_{50}: $\pm 50\,\mu M$) (Passier *et al.,* 1996). Recently, the presence of five locustatachykinins was demonstrated in an extract of 2000 CC of *L. migratoria* by a combination of HPLC and RIA (Passier, 1996). The CA do not stain.

14. Ion Transport Peptide (ITP)

Ion transport peptide has been isolated from aqueous extracts of CC of *S. gregaria* (Audsley *et al.,* 1992a,c). ITP stimulates cl^- transport and causes increases in Na^+, K^+, and fluid reabsorption in the ileum. In addition, it inhibits acid secretion in the ileum (Audsley *et al.,* 1992b). The full sequence of ITP (72 amino acids) and a related peptide (ITP-L) is deduced from cDNAs encoding a 130-amino-acid prohormone for ITP (Meredith *et al.,* 1996). ITP mRNA is restricted to the brain–CC complex, whereas ITP-L is found in many tissues. No immunocytochemical data are available on the distribution of ITP in the brain and CC.

B. Neuropeptides Absent in the Corpus Cardiacum–Corpora Allata Complex (or Whose Presence Has Not Been Demonstrated as Yet)

1. Arginine Vasopressin-like Insect Diuretic Hormone

Two neuropeptides have been isolated from suboesophageal and thoracic ganglia by monitoring HPLC fractions by radioimmunoassay using an anti-arginine vasopressin serum (Proux *et al.,* 1987). F1 is a monomer and F2, which is the biologically active compound, is an antiparallel dimer of F1. The dimer has been shown to display diuretic activity (Proux *et al.,* 1987; Picquot and Proux, 1990) but Coast *et al.,* (1992) have demonstrated that the dimer has no effect on fluid secretion or cAMP levels in locust Malpighian tubules. In the entire central nervous system of *L. migratoria,* only two cells, located in the ventromedial zone of the SOG, give a positive immunoreaction with vasopressin antisera (Rémy and Girardie, 1980; Evans and Cournil, 1990; Tyrer *et al.,* 1993).

2. Kinins

One locustakinin (Lom-KIN) has been isolated from brains of *L. migratoria* (Schoofs *et al.,* 1992b). Locustakinin has no effect on contractions of the hindgut and oviduct of *L. migratoria,* although it is a stimulator of contractions of the hindgut of *L. maderae.* In *L. migratoria,* it stimulates fluid secretion in the Malpighian tubules (Coast, 1995). Kinin-like immunoreactive cell bodies are found in *S. americana* and *L. migratoria* (Nässel, 1993a,b; Chen *et al.,* 1994). Whether kinin-like immunoreactivity is present in the locust retrocerebral complex is not known as yet.

3. Myoinhibin

Locustamyoinhibin (Lom-MIH) has been isolated from brains of *L. migratoria.* It inhibits spontaneous contractions of the hindgut of *L. maderae* and the oviduct of *L. migratoria.* Immunoreactive neurons are found in the optic lobes, the protocerebrum, and the externolateral edge of the tritocerebrum. No perikarya are observed in the SOG or in the ganglia of the ventral nerve cord. Neither the retrocerebral complex nor the neurohemal organs of the ventral nerve cord show immunolabeling (Schoofs *et al.,* 1994).

4. Proctolin

Proctolin is a myotropic peptide that was first isolated from cockroaches (Brown and Starrat, 1975) and later from *L. migratoria* (Schoofs *et al.,* 1993c). Proctolin-like immunoreactivity has been demonstrated in the central nervous system of *Schistocerca nitens* (Keshishian and O'Shea, 1985). No immunoreactivity in the CC or CA has been reported.

C. Processing of Neuropeptide Prohormones

The presence of multiple bioactive peptides within one single precursor is often observed (Douglas *et al.,* 1984). Another important observation in insects is the presence of a multitude of peptides belonging to the same peptide family on the same polypeptide precursor gene (Schneider and Taghert, 1988; Donly *et al.,* 1993; Vanden Broeck *et al.,* 1996). Endoproteolytic enzymes cleave the preprohormones at mono- or dibasic amino acid recognition sequences such as Arg, Lys, Lys-Arg, or Arg-Arg (Sossin *et al.,* 1989; Rehemtulla and Kaufman, 1992). Peptides terminating with a Gly residue immediately prior to the endoproteolytic cleavage site are further modified by removal of the Gly residue to generate an α-amide (Eipper *et al.,* 1992).

1. Adipokinetic Hormone

In *S. gregaria,* two mRNAs encode two adipokinetic preprohormones: prepro-Scg-AKH-1 and prepro-Scg-AKH-2. The prepro-Scg-AKH-1, 63 amino acid residues, contains a signal peptide, Scg-AKH-1, and the α chain. The prepro-Scg-AKH-2, 61 amino acid residues, contains a signal peptide, Scg-AKH-2, and the β chain (O'Shea *et al.,* 1990; O'Shea and Rayne, 1992; Rayne and O'Shea, 1993). The preprohormones are further processed to form pro-Scg-AKH-1 or the A chain and pro-Scg-AKH-2 or the B chain. The prohormone of Scg-AKH-1 is a homodimer of a 41-residue peptide (Fig. 10) (Schulz-Aellen *et al.,* 1989). The A chain, from the N terminus, consists of Scg-AKH-1 followed by a Gly-Lys-Arg processing site and then a 28-residue peptide called the α chain containing a single cysteine and a potential Arg-Lys processing site. The Arg-Lys dibasic pair is not cleaved *in vivo* (Hekimi *et al.,* 1989). *In vitro* studies have revealed the processing of the prohormone of Scg-AKH-1. Endoproteolytic enzymes cleave the precursor at the appropriate processing site (the C-terminal side of Arg^{13} for Scg-AKH-1). Proteolytic processing of C-terminal extended Scg-AKH-1 (Scg-AKH-1-Gly-Lys-Arg) by a carboxypeptidase H-like enzyme removes the basic residues, thereby producing Scg-AKH-1-Gly-Lys, followed by Scg-AKH-1-Gly. A peptidylglycine-α-amidating-monooxygenase produces the amidated bioactive product (Hekimi and O'Shea, 1989; Rayne and O'Shea, 1994).

Recently, the cDNAs encoding the *Locusta* preproadipokinetic hormones (prepro-Lom-AKH-1, 2, and 3) have been isolated and sequenced (Bogerd *et al.,* 1995). The Lom-AKH-1, 2, and 3 precursors are highly homologous to the *S. gregaria* precursors, suggesting the same way of processing, although the precursor of Lom-AKH-3 appears to be more homologous to the AKH/red pigment-concentrating hormone precursors of *Drosophila melanogaster, M. sexta,* and *C. maenas.*

2. FXPRLamides

The amino acid sequence of the Lom-MT prohormone is not known. Arg/Lys-Arg sequences are very common recognition sites for prohormone convertases and a C-terminal Gly is essential for α-amidation. Therefore, it is reasonable to propose the consensus sequence Gly-Arg/Lys-Arg as a recognition site for endoproteolytic cleavage of the locustamyotropin prohormone. These cleaving sites are present in the prohormones of FXPRLamide peptides in *Bombyx mori* and *Helicoverpa zea* (Kawano *et al.,* 1992; Ma *et al.,* 1994). Angiotensin-converting enzyme (ACE) might catalyze the removal of Arg/Lys-Arg from the C terminus of the Lom-MT intermediates generated by the action of prohormone convertases. Indeed,

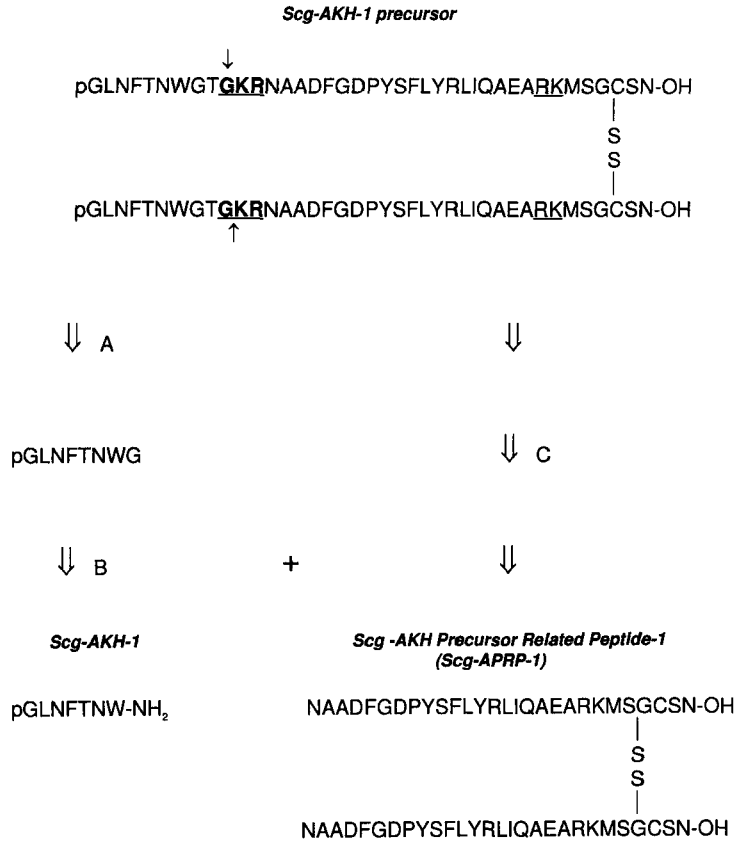

FIG. 10 The precursor of Scg-AKH-1. The prohormone of Scg-AKH-1 is a homodimer of 41 residues. The A chain, from the N terminal, consists of Scg-AKH-1 followed by a **Gly-Lys-Arg** processing site and then a 28-residue region called the α chain containing a single cysteine and a potential Arg-Lys processing site. (A) Endoproteolytic enzymes cleave the precursor at the appropriate processing site (the C-terminal side of Arg13 for Scg-AKH-1). Proteolytic processing of C-terminal extended Scg-AKH-1 (Scg-AKH-1-Gly-Lys-Arg) by a carboxypeptidase H-like activity removes the basic residues producing Scg-AKH-1-Gly-Lys, followed by Scg-AKH-1-Gly. (B) A peptidylglycine-α-amidating-mono-oxygenase activity produces the amidated bioactive product. (C) The Arg-Lys dibasic pair is not cleaved *in vivo*.

recombinant *Drosophila* ACE cleaves the dipeptides Lys-Arg and Arg-Arg from the C terminus of FSPRLGKR and FSPRLGRR, respectively, to yield FSPRLG. The generated FSPRLG is relatively resistant to further hydrolysis by ACE, allowing the Gly extended peptide to serve as a substrate for a peptidylglycine α-amidating monooxygenase. Neurosecretory cells in the brain and suboesophageal ganglion that contain Lom-MT-like

material are also immunoreactive for ACE (Fig. 12). The coexistence of ACE and Lom-MTs within locust neurosecretory cells suggests a role for ACE in intracellular processing of the Lom-MT prohormone (Isaac *et al.*, 1997b).

3. Insulin-Related Peptide

The gene of Lom-IRP has been cloned and is present as a single-copy/haploid genome. The organization of the gene is similar to that of mammalian insulin genes and the molluscan insulin gene (Smit *et al.*, 1993), but it is different from the large number of intronless insulin genes, more than 30/haploid genome, which have been found in *B. mori* (Nakakura *et al.*, 1992). There are at least two Lom-IRP transcripts. Lom-IRP-T1 is only expressed in neurosecretory cells of the brain while Lom-IRP-T2 is present at low levels in several tissues. The Lom-IRP gene has at least two promoters, the alternative usage of which accounts for a differential regulation of expression of Lom-IRP in neurosecretory cells and in other tissues (Kromer-Metzger and Lagueux, 1994). In neurosecretory cells of the brain, a 145-residue precursor is produced, which is posttranslationally processed into a 21-residue A chain, a 31-residue B chain, and a C peptide (Lageux *et al.*, 1990).

4. Ion Transport Peptide

A putative ion transport peptide (Scg-ITP) prohormone can be cleaved at two dibasic amino acid sites to yield the 72-residue active amidated peptide. The mRNA for Scg-ITP is restricted to the brain and CC (Meredith *et al.*, 1996).

5. Schistostatins

The cDNA encoding the precursor polypeptide for schistostatins has been cloned. It encodes 10 schistostatins, 2 of which have not been isolated (Vanden Broeck *et al.*, 1996). The schistostatin precursor differs from that of cockroach preproallatostatins in size, in sequence, and in organization. It contains a lower number of peptides that are interrupted only once by an acidic spacer region. It has been suggested that acidic regions are needed to compensate for the many basic residues that are present in the cleavage sites of prepro-AST precursor polypeptides. In agreement with a reduction of the number of acidic regions, there is indeed a lower number of AST-like peptides in the schistostatin precursor compared to that in the cock-

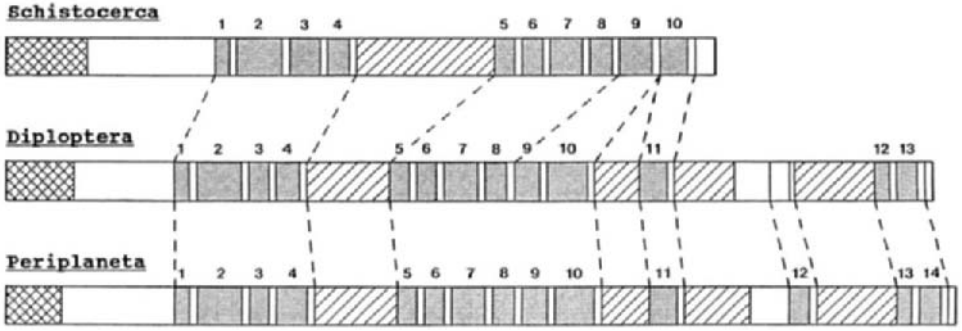

FIG. 11 Scheme showing a comparison of the organization of AST-precursor polypeptides from the desert locust *Schistocerca gregaria* and from two distantly related cockroach species, *Diploptera punctata* and *Periplaneta americana*. The secretory signal peptides are shown as cross-hatched boxes. AST-like peptide sequences are shown as small shaded boxes. These are numbered according to their position in the precursor. Acidic spacer regions, which interrupt the peptide encoding portions of the precursors, are represented by hatched boxes. The schistostatin precursor is shorter than the cockroach allatostatin precursors, contains a lower number of AST-like peptides, and has only one single acidic spacer.

roach ones (Fig. 11). Why so many schistostatins? Each of the 10 peptides of the schistostatin family has a unique N-terminal address sequence. These individual address sequences may regulate recognition and strength of binding to one or more receptors or could modulate the stability of the molecules and hence the duration of their activity. The acidic domain within the precursor effectively separates the individual schistostatins into two groups: schistostatins 1–4 and schistostatins 5–10. Such a separation may suggest some functional significance such as synergism between individual peptides within groups or separate timing of release of peptide groups, or it may reflect groups targeted for discrete functions.

D. Second Messengers

Although some peptides have been isolated using cAMP assays (Clottens *et al.*, 1995; Proux and Herault, 1988; Lehmberg *et al.*, 1991; Kay *et al.*, 1991; Veelaert *et al.*, 1995c), not that much is known about the signal transduction of neuropeptides involved in the release of adipokinetic hormone and juvenile hormone in locusts.

1. Adipokinetic Hormone

The involvement of cAMP as a second messenger in the control of adipokinetic hormone release has been well established (Orchard and Lange, 1983a;

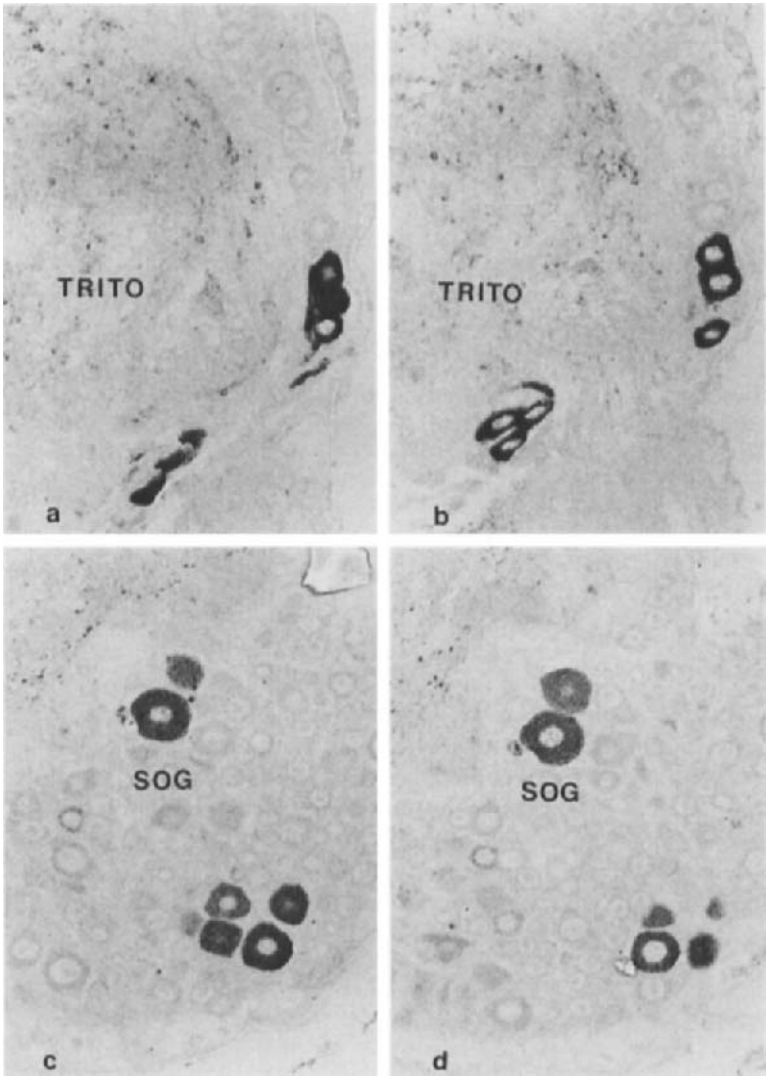

FIG. 12 Consecutive sections showing colocalization of ACE- and Lom-MT-like material. Consecutive sections of the tritocerebrum of the brain of *Locusta migratoria* showing colocalization of (a) ACE- and (b) Lom-MT-like material (original magnification, ×1000). Consecutive sections of the suboesophageal ganglion of *Locusta migratoria* showing colocalization of (c) ACE- and (d) Lom-MT-like material (original magnification, ×1000).

Orchard *et al.*, 1983; Passier *et al.*, 1995). Recently, it has been demonstrated immunocytochemically that the NCC II contains Lom-TKs and FMRF-amide related peptides (FaRPs) (Nässel *et al.*, 1995; Passier *et al.*, 1994). Until now, five Lom-TK analogs have been isolated from *L. migratoria* brain–CC–CA complexes (Schoofs *et al.*, 1993c). Recently, all five known Lom-TK analogs have been isolated from the CCg (Passier, 1996). The adipokinetic hormone cells in the CCg respond to a stimulus of 100 μM Lom-TK-1 with a rapid elevation of their cAMP levels (Nässel *et al.*, 1995). A similar stimulus of Lom-TK-1 initiates the release of Lom-AKH-1 in a dose-dependent manner (Nässel *et al.*, 1995). Lom-CCAP, also a releasing factor of adipokinetic hormone, does not significantly increase cAMP levels in AKH cells (P. C. C. M. Passier, personal communication).

Two different FaRPs, namely, FMRFamide and SchistoFLRFamide (Scg-FLRFamide-1), both inhibit an induced release of AKH *in vitro* (Passier *et al.*, 1994). Application of IBMX induces a marked increase in the release of AKH while Scg- FLRFamide-1 significantly reduces this IBMX-induced release of AKH (Passier, 1996).

Next to neuroactive substances, humoral factors can play a role in the release of AKH as well. It is known that resting levels of trehalose (80 mM) in the hemolymph prevent the release of AKH, whereas flight levels (40 mM) have no effect (Cheeseman *et al.*, 1976; Van der Horst *et al.*, 1979). Another humoral factor effecting the release of AKH might be the stress hormone octopamine. Although Orchard and co-workers (1993; Pannabecker and Orchard, 1986) suggested that octopamine might act as a neurotransmitter, it cannot be detected either immunocytochemically or electrochemically in the CCg (Konings *et al.*, 1988a; Passier *et al.*, 1995). Moreover, in the absence of a cAMP-enhancing agent, such as IBMX, it has no effect on AKH release. In the presence of IBMX, however, it enhances the IBMX-induced release of AKH. At the onset of flight, the octopamine titer in the hemolymph is three times higher than that during stress (Goosey and Candy, 1980). Therefore, octopamine is rather a humoral factor affecting the release of AKH.

2. Juvenile Hormone

Today, no neuropeptide has been reported to affect the release of juvenile hormone by the CA in the locust. Schistostatins (Scg-ASTs) inhibit the phosphorylation of a protein in the CA of the locust (Veelaert *et al.*, 1996c). In the cockroach, allatostatins seem to change the cAMP and cGMP levels in the CA (Cusson *et al.*, 1992b; Stay *et al.*, 1994).

E. Inactivation and Metabolism of Neuropeptides

The inactivation of intracellular signal molecules must be viewed as a complex process extending from the point of receptor activation by these

molecules to their clearance from the area bearing their products. Processes of diffusion away from the receptor, concentrative reuptake, extra- and intracellular enzymatic hydrolysis or covalent modification, and internalization in association with a receptor, or combinations of these processes, are possible mechanisms by which neuropeptides could be inactivated (McKelvy and Blumberg, 1986). Most of the studies on the inactivation of insect neuropeptides have to do with their enzymatic degradation.

1. Adipokinetic Hormone

Since AKHs possess chemically blocked N and C termini which provide protection from most amino and carboxy peptidases, enzymatic inactivation of these peptides would be expected to be initiated by an endopeptidase. Indeed, neural membranes from ganglia of the locust *S. gregaria* contain peptidase activity, which hydrolyzes Scg-AKH-1 to generate pGlu-Leu-Asn and Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2 as primary metabolites (Isaac, 1988). It has been suggested that in the hemolymph AKH is degraded by intracellular enzymes following internalization by nonneural tissues (Mordue and Stone, 1978; Fox and Reynolds, 1991).

Homogenates of Malpighian tubules of *S. gregaria* cleave Scg-AKH-1. Three degradation products have been demonstrated: pGlu-Leu-Asn-Phe-Thr-Pro, Trp, and Trp-Gly-Thr-NH_2 (Siegert and Mordue, 1987, 1992a,b). Inactivation of Lom-AKH-1 and Lom-AKH-2 in the circulation is achieved by an endopeptidase present on the surfaces of the fat body, Malpighian tubules, and skeletal muscles. The enzyme cleaves at the Asn-Phe bond to generate pGlu-Leu-Asn, Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2, and Phe-Ser-Thr-Gly-Trp-NH_2. None of these fragments display adipokinetic activity (Rayne and O'Shea, 1992).

2. Angiotensin-Converting Enzyme

In a previous section we reported that ACE is able to process pro-Lom-MT peptides (Isaac *et al.*, 1997b). ACE is also able to degrade other peptides. Lom-TK-1, Lom-TK-2, Lom-AKH-1, Scg-FLRFamide-1, and Dip-Ast-7 are hydrolyzed by housefly angiotensin-converting enzyme, while CCAP and proctolin were not suitable substrates. Lom-TK-1 is cleaved at the C-terminal side to release a dipeptide amide as major fragment (Lamango *et al.*, 1997). The ability of insect ACE to hydrolyze C-terminally amidated peptides might be of functional significance because the enzyme has been localized in neuropile regions of the locust brain (Isaac *et al.*, 1997a). Analogs of insect kinins containing aminoisobutyric acid (Aib) proved to be resistant to hydrolysis by housefly ACE. The Aib residue is

compatible with formation of a turn in the active core region that is important for the biological activity of insect kinins (Nachman *et al.*, 1997).

3. Arginine Vasopressin-like Insect Diuretic Hormone (AVP-like IDH)

In *L. migratoria,* the transformation of AVP-like IDH of the monomer to the dimer starts in the suboesophageal ganglion and is enzymatically controlled. The monomer and dimer are simultaneously released from the suboesophageal ganglion into the hemolymph, where the transformation of monomer to dimer continues. Finally, the dimer is transported to the Malpighian tubules, where it is enzymatically degraded (Picquot and Proux, 1990).

4. Proctolin

Neural homogenates degrade proctolin at neutral pH by cleavage at the Arg-Tyr and Tyr-Leu bonds to yield Tyr-Leu-Pro-Thr, Arg-Tyr, and free tyrosine. Arg-Tyr has been detected as a major metabolite in the presence of aminopeptidase inhibitors to prevent Arg-Tyr breakdown (Isaac, 1987). The aminopeptidase activity is concentrated in the synaptic membrane preparation. The subcellular localization of the membrane aminopeptidase is consistent with a possible physiological role for this enzyme in the inactivation of synaptically released proctolin (Isaac, 1987). Similar results have been obtained in the American cockroach *P. americana* (Quistad *et al.,* 1984). Proctolin is also degraded by ovary and hindgut preparations of *L. migratoria* (Puiroux and Loughton, 1992). The pH is critical for enzymatic activity. Inactivation of proctolin at different pHs revealed that three primary hydrolytic pathways account for the *in vitro* metabolism of proctolin in *P. americana.* The major primary cleavage site at pH 6 is the Pro-Thr bond and at pH 8 it is the Tyr-Leu bond; at both pHs cleavage also occurs at the Arg-Tyr bond. Several secondary and tertiary degradation pathways have also been found (Steele and Starratt, 1985).

5. Schistostatins

The most simple definition of neuropeptide inactivation by enzymatic hydrolysis is that the action of the enzyme results in the generation of products that cannot effectively activate a particular receptor. Scg-AST-2 and Scg-AST-2^{11-18}, which is probably a degradation product of Scg-AST-2 generated by endopeptidase activity, are two neuropeptides purified from a brain extract. Scg-AST-2 is the most potent inhibitor of JH biosynthesis by the CA of the cockroach *D. punctata,* while Scg-AST-2^{11-18}, which has the same

active core as all schistostatins, seems to be a stimulator of JH biosynthesis (Veelaert *et al.*, 1996b; Schoofs *et al.*, 1997b).

The half-life of Dip-AST-7 in cockroach hemolymph is 21.8 min. Dip-AST-7 is degraded into two products, with amino acid compositions corresponding to Leu-Tyr-Gly-Phe-Gly-Leu-amide and to Tyr-Gly-Phe-Gly-Leu-amide. Similarly, the half-life of Dip-AST-9 in cockroach hemolymph is 22.3 min. It is degraded into two products, Leu-Tyr-Ala-Phe-Gly-Leu-amide and Tyr-Ala-Phe-Gly-Leu-amide (Garside *et al.*, 1997). These two products represent the C-terminal message sequence of allatostatins and their presence suggests that, as might be expected, degradation of the peptides occurs through the action of an aminopeptidase (Stay *et al.*, 1994). It is expected that a metalloendopeptidase in the hemolymph cleaves Dip-AST-7 and Dip-AST-9 at Arg-Leu to yield the C-terminal hexapeptide. This hexapeptide is subsequently cleaved by an amastatin-sensitive aminopeptidase (Garside *et al.*, 1997). Surprisingly, allatostatins occur in the hemolymph (Woodhead *et al.*, 1993; Yu *et al.*, 1993) despite the existence of mechanisms for their degradation.

F. Structure–Activity Studies

The isolation of a high number of naturally occurring neuropeptides belonging to certain families with a specific consensus sequence suggests that the consensus sequence acts as the active core and *message* portion of those neuropeptides. In contrast, the N-terminal region or the *address* sequence of the molecules shows considerable variation. Although many neuropeptides have been purified, not very much is known about their receptors (Vanden Broeck, 1996). The design of neuropeptide analogs with modifications in the active core sequence can yield a lot of information about the peptide–receptor interaction. The analogs represent a milestone in the development of nonpeptide mimetic analogs. Mimetic analogs are potentially valuable tools to insect neuroendocrinologists studying these physiological processes and/or engaged in the development of future pest management strategies. In this part we describe the activity of synthetic peptide analogs in various bioassays.

1. Adipokinetic Hormone

Replacement of the hydroxyl hydrogen in Thr^5 of Lom-AKH-1 by the bulky and highly lipophilic tert-butyl group reduces the potency markedly, whereas the efficacy is unaffected. Replacing the hydroxyl hydrogen of Thr^{10} in Lom-AKH-1 by a benzyl group does not alter the activity of the resulting analog compared to that of the natural peptide (Poulos *et al.*,

1994). Analogs of Lom-AKH-1 with modifications at the C-terminal threonine residue have been evaluated in both an *in vivo* lipid mobilization assay and an *in vitro* acetate uptake assay. Modifications at Thr^{10} of Lom-AKH-1 involve the replacement of its C-terminal amide by the groups OH, OCH_3, $NHCH_3$, $N(CH_3)_2$, and NHC_6H_5. Uptake of acetate has a greater preference for a hydrophobic C terminus than lipid mobilization (Lee *et al.*, 1996). Lom-AKH-1^{4-10}, purified from 900 brains of *L. migratoria,* has myostimulatory activity but no adipokinetic activity (Schoofs *et al.*, 1993b).

2. FLRFamide Peptide Family

In the locust, oviduct two receptors have been revealed by a binding assay, a high-affinity receptor associated with the upper lateral oviducts, which receives little or no innervation, and a low-affinity receptor associated with the lower lateral and common oviducts, which receives extensive innervation (Wang *et al.*, 1994).

Substitution of Gly for Pro^3 in the heptapeptide pQDPFLRFamide significantly improves the characteristic FMRFamide-like biological activity in the extensor tibiae neuromuscular preparation of the locust. Substitution of Asn for Asp^2 and aminoisobutyric acid for Pro^3 are conservative with respect to the heptapeptide biological activity (Geraghty *et al.*, 1994).

The His residue in the truncated analog HVFLRFamide of SchistoFLRFamide (Scg-FLRFamide-1) is critical for the retention of biological activity in the oviduct of *L. migratoria,* whereas VFLRFamide, in which biological activity is lost, is the minimum sequence for binding with comparable affinity to the parent compound. When the His residue in HVFLRFamide is substituted by Tyr, Leu, Ile, or Val, these hexapeptide analogs exert stimulatory effects on oviduct muscle contraction. In this system, inhibitory peptides and stimulatory peptides share a single receptor by having the same binding sequence, VFLRFamide, but are able to produce opposite muscle responses due to differences in activation sites. Correspondingly, this single receptor could be coupled with two different intracellular signaling systems to mediate either inhibitory or stimulatory responses (Wang *et al.*, 1995d). The C-terminal RFamide group is critical for binding affinity and biological activity of analogs and substitution of the C-terminal Arg or Phe with the structurally similar amino acids Lys or Tyr results in two high-affinity antagonists (Wang *et al.*, 1995b). Substitution of the His by the D isomer or Phe changed the activity dramatically, suggesting that the ring of histidine may be required for inhibitory activity (Lange *et al.*, 1996). The N terminal appears to play little or no role in either binding or activation (Wang *et al.*, 1995c). Receptors for the inhibitory and stimulatory FaRPs are coupled with G proteins and both inhibitory and excitatory effects of FaRPs on locust oviducts are mediated through the activation of G proteins.

This implies that the receptor is very likely coupled to two different G proteins, one for the inhibitory effect and the other for the stimulatory effect (Wang *et al.,* 1995a).

Benzethonium chloride (Bztc), which is a nonpeptide ligand for the FLRFamide receptor family, mimics the physiological effects of SchistoFLRFamide (Scg-FLRFamide-1) on locust oviduct. Bztc competitively displaces SchistoFLRFamide, binding to both high- and low-affinity receptors of membrane preparations (Lange *et al.,* 1995). The activity of a neuropeptide (leucomyosuppressin) and of the nonpeptide Bztc on muscle contractions can be blocked by the lipoxygenase inhibitor nordihydroguaiaretic acid, providing evidence for a similar mode of action. In addition, Bztc competitively displaces a radiolabeled myosuppressin analog from high- and low-affinity receptors of the locust oviduct. Thus, the nonpeptide interacts with both binding and activating regions of myosuppressin receptors (Nachman *et al.,* 1996a).

3. FXPRLamides

The biological active core of FXPRLamides is Phe-Thr-Pro-Arg-Leu-NH_2. Lem-Pk with a free C terminus exhibits little activity in a myotropic assay. Within this core sequence, Thr at position 4 from the C terminus is tolerant for amino acid substitution without substantial loss of biological activity. A type I β turn structure resides in the sequence between Thr^5 and Leu^8 of Lem-PK (pGlu-Thr-Ser-Phe-Thr-Pro-Arg-Leu-NH_2). This structure is supported by a hydrogen bond between the Thr^5 carbonyl oxygen and the Leu^8 amide proton (Nachman *et al.,* 1991). The C-terminal β turn is the active conformation recognized by the myotropic receptor.

Cbe-Thr-Pro-Arg-Leu-NH_2 (Cbe: 2-*o*-carboranylethanoyl), a pseudotetrapeptide analog of FXPRLamide, in which the phenyl ring of the Phe side chain is replaced by the hydrophobic cage-like *o*-carborane moiety, is very potent in the cockroach hindgut bioassay system. Its effect cannot be immediately reversed following a saline rinse, providing evidence that the analog binds very strongly to the receptor (Nachman *et al.,* 1996b). Dipeptide analogs of FXPRLamide containing a trans-DL-1,2-cyclopentanedicarboxyl carbocyclic component approached the potency of the pentapeptide core in a cockroach hindgut bioassay system (Nachman *et al.,* 1995).

4. Kinin

A combined experimental and theoretical analysis of the insect kinin carboxy-terminal pentapeptide has been used to probe the role of each residue, define bioactive conformation, and design a constrained bioactive analog. Receptor-binding and signal transduction assays reveal that each

residue of the insect kinin carboxy-terminal pentapeptide has a distinct role in conformational preference, specific receptor interactions, or signal transduction. The β turn preference of residues Phe(1) X(2) X(3) Trp(4) implicates this as the bioactive conformation. The amidated carboxyl terminus, required for activity in many neuropeptide families, may be generally important for signal transduction and its inclusion may therefore be essential for agonist design (Roberts *et al.,* 1997). One analog containing Aib, pGlu-Lys-Phe-Phe-Aib-Trp-Gly-NH_2, is more active than most active endogenous insect kinins in cockroach hindgut myotropic and cricket Malpighian tubule fluid secretion assays (Nachman *et al.,* 1997). The analog also shows higher resistance against degradation by the angiotensin-converting enzyme. This analog, blocked at both the amino and the carboxyl termini, is less easily degraded in the hemolymph than endogenous peptides.

5. Proctolin

The pentapeptide structure of proctolin is necessary for its activity and modifications decrease its potency (Starratt and Brown, 1979). Tripeptides, Arg-Tyr-Thr, Tyr-Arg-Thr, and Leu-Pro-Thr, show no agonistic effect on the foregut of *S. gregaria,* with Arg-Tyr-Thr being able to antagonize proctolin-induced contractions (Gray *et al.,* 1994). Substitution of Tyr^2 for Trp^2 decreases its activity, suggesting that the aromatic group of Tyr^2 is necessary for activity (Sullivan and Newcomb, 1982). [*α-methyl*-L-tyrosine^2]Proctolin proves to be a potent antagonist of proctolin response, whereas [*N-methyl*-L-tyrosine^2]proctolin shows no agonistic or antagonistic effects and cycloproctolin shows a weak antagonist effect on the foregut of *S. gregaria* (Gray *et al.,* 1994). Both [*α-methyl*-L-tyrosine^2]proctolin and [*N-methyl*-L-tyrosine^2]proctolin are agonists of proctolin in the locust oviduct bioassay. At threshold doses, both molecules are antagonists when administered together with proctolin. The tripeptides Arg-Tyr-Thr, Tyr-Arg-Thr, and Leu-Pro-Thr show minimal agonistic effects and cycloproctolin antagonizes proctolin's response in the locust oviduct bioassay (Noronha *et al.,* 1997). Proctolin analogs display differential cardioexcitatory effects in the cockroach (Bartosz-Bechowski *et al.,* 1990; Konopinska *et al.,* 1986, 1988, 1990).

Modifications in Arg^1 and Tyr^2 position result in analogs with decreasing potency (Lange *et al.,* 1993). A decrease in chain length or an increase in hydrophobicity decreased bioactivity on the locust hindgut and oviduct but not necessarily binding. A significant discrepancy between the high binding capacity of the proctolin analog Arg-Tyr-Ser-Pro-Thr and its relatively low myotropic activity has been reported (Puiroux *et al.,* 1993). Ser^3-proctolin has a higher affinity for membranes but is less active, suggesting that the leucine in position 3 of proctolin is more important for bioactivity than for

binding in both oviduct and hindgut (King *et al.,* 1995). The threshold concentration for biological activity on the *L. migratoria* oviduct of Ala^1-proctolin is 1000 times higher compared to that of proctolin itself (Spittaels *et al.,* 1995). Proctolin analogs, containing D-amino acids, show high agonistic activity in the cardioexcitatory test on the heart of the yellow mealworm *Tenebrio molitor* (Kuczer *et al.,* 1996). There are two proctolin receptor subtypes in locust oviduct membranes (King *et al.,* 1995). The action of proctolin on the locust extensor tibiae muscle is mediated by at least two receptor subtypes (Baines *et al.,* 1996).

6. Schistostatins

The C-terminal sequence part (FGLamide) is well conserved in all peptide family members and might be part of the active core. AST analogs have been examined for their ability to reduce JH biosynthesis in the CA of the cockroach *D. punctata.* Deamidation of the C-terminal Leu, modification of the C terminus, through either truncation (of Leu or Gly-Leu) or extension (addition of Ala or Ala-NH_2) abolishes activity with respect to inhibition of JH biosynthesis (Pratt *et al.,* 1989, 1991). Ala or D-amino acid replacements have been introduced in two series of Dip-AST-5 analogs. Tyr^4, Phe^6, and Leu^8 are the three most important residues for both potency and efficacy and the conformation of the C-terminal tripeptide is likely to be a type II β turn centered around the Phe^6-Leu^8 position (Hayes *et al.,* 1994).

Two Dip-AST-7-specific proteins of molecular size 59 and 39 kDa have been demonstrated in CA membranes (Cusson *et al.,* 1991, 1992a). For Dip-AST-2 and Dip-AST-4 a putative receptor (37 kDa) has been demonstrated and partially characterized in brain and CA of the cockroach *D. punctata* (Yu *et al.,* 1995). Membrane preparations derived from other tissues also result in specific binding of certain protein bands in brains (41 kDa) and fat body (38, 41, and 60 kDa) in *D. punctata* (Cusson *et al.,* 1991, 1992a,b).

7. Tachykinins

Lom-TKs have a Phe residue at position 5 from the C terminus. The Gly residue at position 3 from the C terminus is likely to form a type I β turn supported by an intramolecular hydrogen bond between the Tyr or His carbonyl oxygen and the C-terminal amide proton (Nachman *et al.,* 1991).

IV. Concluding Remarks

For quite a long time it was believed that insects, when compared to vertebrates, have a simple endocrinological system. Today, however, more

than 50 neuropeptides have been isolated and identified from the nervous system of locusts. Although it is clear that many of these neuropeptides have defined functions, including regulation of reproduction, development, pigment biosynthesis, and osmoregulation, as well as myotropic actions and behavioral effects (Schoofs *et al.*, 1996), almost no information is available about the possible role of these neuropeptides as a neurotransmitter and/or neuromodulator in the locust brain.

The innervation of the locust retrocerebral system seems to be very elaborate. Many neurosecretory cells of the brain and the suboesophageal ganglion have axons projecting to this system. Immunocytochemical localization of the identified locust peptides in the retrocerebral organs and in the neurosecretory cells projecting to these organs can provide useful information as to which peptides are good candidates for having a function in the control of secretory activity of the retrocerebral complex. Neuropeptides occurring in the nerves projecting to the retrocerebral complex should be tested for regulation of secretory activity. In addition, new brain extracts should be screened in reliable releasing assays and purified in order to isolate and identify insect releasing factors. Until now, only one releasing factor (CCAP) has been identified in insects. This is the first releasing factor ever identified in invertebrates.

A lot of work remains to be done in the study of the processing of precursor polypeptides. In insects, precursors have been shown to contain up to 13 copies of 1 peptide or 13 analogs of the same peptide. The reason for this is not known but might be to increase the amount of peptide produced from one strand of mRNA. Extensive use of molecular biology techniques will sort out this question. Molecular biology techniques can also contribute a great deal to the isolation of neuropeptides, as has been shown for *Hydra magnipapillata,* from which 286 peptides have been isolated using differential display–PCR as a screening method (Takahashi *et al.*, 1997).

In sum, insect (invertebrate) neuroscience has advanced into research areas previously dominated by vertebrate neuroscience. These research disciplines have revealed that the insect nervous system is much more complicated than previously thought.

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