LOCALIZATION AND CHARACTERIZATION OF AN ECTODERMAL PROTEIN OF SEA URCHIN EMBRYOS

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A LA MEMOIRE DE MON PERE

ABSTRACT

In the urchin Strongylocentrotus purpuratus, the expression of the Spec3 gene is associated with the growth of cilia, one of the first morphogenetic events during development The product of this gene was characterized using an antiserum raised against a peptide corresponding to the predicted amino-terminal portion of the protein This thesis describes the localization of the Spec3 protein at different stages during embryonic development Immunocytochemistry indicated that the protein is associated with cilia and Golgi complexes of ectodermal cells Agents that inhibit protein synthesis and Golgi secretion also altered its normal distribution Fractionation of cilia and immunoblotting indicate that the protein is associated with the ciliary axoneme and that it behaves as a large aggregate.

RESUME

Chez l'oursin Strongylocentrotus purpuratus, l'expression du gene Spec3 est associee à l'apparition de cils caractéristiques du debut de la morphogenese Pour etudier le produit de ce gène, j'ai utilise un antiserum obtenu à partir d'un peptide correspondant à l'extremite amino de la sequence presumée de la protéine. Dars cette these, je decris la distribution de la protéine Spec3 dans l'embryon a différents stades de son développement. L'analyse immunocytochimique revele que la proteine se retrouve dans les cils et l'appareil de Golgi des cellules de l'ectoderme Sa distribution est pertubee par des drogues qui inhibent la synthèse des proteines et la secretion à partir de l'appareil de Golgi. Le fractionnement des cils et l'immunotransfert après électrophorèse des differentes fractions revele que la protéine Spec3 est associee à l'axonème des cils et qu'elle se comporte comme un aggregat

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PREFACE

This thesis is a prolongation of the work that Elizabeth Dolliver Eldon started in W.H. Klein's laboratory In her Ph D thesis, she described the structure and expression of the Spec3 gene She also raised an antibody against a peptide corresponding to the predicted N-terminal region of the protein She used this antibody to characterize the localization of the Spec3 protein in the sea urchin embryo. During a sabbatical leave, Dr Bruce Brandhorst, my thesis supervisor, started to do experiments on Spec3 using drugs such as monensin and colcemid. I will describe their results in more details in the introduction to this work.

During the course of my work, I made several observations that added new elements to previous knowledge on the Spec3 protein.

I localized Spec3 on cilia in the ciliary band of pluteus larvae and observed a structure in ciliary band basal cells that reacts with the Spec3 antiserum as well as with an anti-tubulin antiserum Using ethidium bromide to stain nuclei, I confirmed that Spec3 is present in each ectodermal cell, residing in the Golgi complex, a structure that lies in close apposition to the nucleus I characterized in detail the distribution of Spec3 in blastulae and correlated changes in distribution of Spec3 with the patterns of movement at the pre-hatching, hatching and mesenchyme blastula stages I also described the distribution of Spec3 in 16 cell stage embryos and metamorphosing larvae

I analyzed the effect of monensin and deciliation on ciliary growth and the localization of Spec3. I found that monensin inhibits ciliary regeneration under certain conditions

I found that inhibition of protein synthesis causes an accumulation of Spec3 at the tip of cilia and that this effect is reversible.

By immunoblotting, I showed the enrichment of Spec3 in cilia and the accumulation of Spec3 in cilia during embryonic development I fractionated ciliary proteins in several ways and showed that the Spec3 protein is strongly associated with the insoluble fraction of the axoneme.

Part of the work reported in this thesis has been accepted for publication in the journal Genes and Development under the following title:

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My labmates created a friendly atmosphere for work Francis Ouellette was wonderfully supportive and encouraged me to persist as I was struggling with the unfamiliar concepts of molecular biology He also gave useful suggestions for the final version of this thesis I had interesting and stimulating conversations with Dr Mario Filion about my project and science in general. Fang Hung, Arianna Lee and

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INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The study of development is as old as the science of biology. Developmental biologists are still trying to answer the fundamental question of their discipline how does a single cell, the egg, change to become an organism with many cell types and functions.

Over the centuries, the theoretical frames used by biologists influenced their approach to the study of development (Moore, 1987, Oppenheimer, 1971) For example, the popular question of epigenesis *versus* preformation raised much activity among embryologists who were trying to find out whether the adult organism is already present in miniature in the egg or is shaped as development proceeds In the absence of appropriate experimental tools, the argument remained theoretical for many centuries

The publication of Darwin's theory of evolution popularized the concept of recapitulation. Embryology put aside mechanistic interrogations to search for as many natural histories as possible in order to validate the notion that "Ontogeny recapitulates phylogeny"

The ability to perform experiments on embryos generated many questions on the mechanisms of development. Following the production of fate maps for different embryos, the concept of cell determination was investigated. Several lines of evidence showed that the fate of certain cells in some embryos is determined and cannot be changed. The fate of other cells depends on external factors and can be changed by varying those factors Certain cells influence the fate of their neighbors, a process called induction.

The understanding of genetic inheritance and the availability of molecular tools in biology has given rise to a current model for development: cell differentiation is the result of differential temporal and spatial gene activity (Davidson, 1986) Cells become different because they express different genes. In that context, it is important to identify genes whose activity varies in space and time during development. The Spec3 gene of sea urchins, whose product is the subject of this thesis, is such a gene. Its transcript appears in the embryo just before the differentiation of the ectoderm and it becomes localized exclusively in ectodermal cells

The fact that the expression of the Spec3 gene is intimately related to ectodermal differentiation raises the question of its function Eldon et al (1987) found that its expression is associated with the appearance of cilia

I wanted to investigate the characteristics of the Spec3 protein It was found that it is present on cilia and in the Golgi apparatus of ectodermal cells (Eldon et al, 1989) I made more detailed observations on the localization of the Spec3 protein in different cell types at various stages of development, I looked at the effect of drugs that perturb protein metabolism on the localization of the Spec3 protein, and I fractionated isolated cilia to determine where the protein is located in the ciliary structure

LITERATURE REVIEW

Sea urchin development

The developmental program of the sea urchin Strongylocentrotus purpuratus is aimed at the formation of a mature pelagic ciliated larva that eventually metamorphoses into an adult urchin Most of the embryonic structures disintegrate at metamorphosis when the adult urchin emerges from a rudiment formed inside the larva Most of what is known about sea urchin development relates to the embryonic and larval stages Programs of gene expression after metamorphosis have not been studied yet.

Echinoid development has been reviewed by many authors The following description is based on Czihak (1971), Giudice (1973, 1986) Davidson (1986) and Wilt (1987).

The egg of the sea urchin Strongylocentrotus purpuratus is about 80 μm in diameter and contains a store of mRNAs and proteins sufficient to allow the embryo to develop until it is able to start feeding. After fertilization, a tough envelope rises around the zygote The first two cleavages take place along the pre-localized animal-vegetal axis. The third cleavage is equatorial The fourth cleavage is meridional in the blastomeres from the animal half, giving rise to 8 mesomeres but in the vegetal half, the plane of cleavage is parallel to the equator and separates the blastomeres inequally into 4 small micromeres at the vegetal pole and 4 macromeres adjacent to the mesomeres

Cell division is synchronous until the 9th or 10th cleavage except for the micromeres whose cleavage is delayed with respect to

other blastomeres. The 4 micromeres undergo one cell division One daughter cell enters the primary mesenchyme skeletogenic lineage whereas the other divides once more and then exits the cleavage program. Much later, at the feeding larva stage, it resumes cell division to form a part of the coelomic sac constituents which give rise to the imaginal rudiment of the adult urchin (Pehrson and Cohen, 1986).

When the cleavage becomes asynchronous, the embryo is a hollow ball of cells, the blastula Ciliogenesis begins in all cells except the 8 micromeres that have stopped dividing after the 6th cleavage In some species, the micromeres are also ciliated (Masuda, 1979) The formation of cilia allows the embryo to spin inside the fertilization envelope At the animal pole , some cilia grow longer than the others, forming the apical tuft The blastula synthesizes and secretes a protein, the hatching enzyme, which digests the fertilization envelope, liberating the swimming blastula in sea water

After hatching (or sometimes before), the vegetal region flattens, forming the vegetal plate In this region of the embryo, some cells lose their cilia and begin to migrate in the blastocoel These mesenchyme cells eventually form the embryonic skeleton At that stage, there are two germ layers the ectoderm on the external surface and the mesoderm comprising the mesenchymal cells

Invagination of the vegetal plate initiates gastrulation Invagination and reorganization of cells result in the formation of the archenteron, the endoderm At the tip of the forming archenteron, secondary mesenchyme cells are continuously released They are the

precursors of the pigment cells of the larval epithelium, the esophagal muscle and some of the coelomic sac constituents, some of which give rise to the adult rudiment.

As the primitive gut reaches to one side of the blastocoel, the mouth forms where it touches the ectodermal wall. The other end of the archenteron becomes the anus, a characteristic of Deuterostomes. As the larval skeleton develops, the shape of the embryo changes Four arms form around the mouth, framing the oral area. As oral arms grow, the apical tuft of cilia disappears. The opposite side of the embryo, the aboral end, elongates and becomes pointed at the apex The larva looks like an easel, whence its latin name pluteus. It feeds on phytoplankton and small suspended particles (Strathman, 1975)

The oral field of the pluteus is surrounded by the ciliary band. Each cell of this band has one motile cilium (Strathman, 1975, Nakajima, 1986a) In the preoral area, additional coiled cilia are embedded in the hyaline layer at the surface of epithelial cells (Nakajima, 1986a)

When a food particle reaches the ciliary band, several cilia change their pattern of beating to bring the food to the mouth When food particles are retained by the ring of cilia that immediately surrounds the mouth, they are eaten readily (Strathman, 1975)

Larvae can swim backward by reversing their ciliary beat along most of the ciliary band (Strathman, 1975) Such reversal of ciliary movement can be triggered by stimulation of the larva. During ciliary reversal, electrical activity can be recorded. Moreover, magnesium chloride, a suppressor of nervous activity in invertebrates, prevents

ciliary reversal from taking place Thus, ciliary reversal is associated with nervous activity (Mackie et al, 1969). Cells of the ciliary band are associated with axonal bundles and neural receptor cells (Burke, 1978,1983, Nakajima, 1986b)

In some urchin species, parts of the ciliary band separate from the rest of the band. These epaulettes are involved in locomotion rather than particle capture (Strathman, 1975)

The adult rudiment develops from the union of a small part of the ectoderm and one of the coelomic pouches adjacent to the gut (Hinegardner, 1975) When the larva is ready to metamorphose, after several weeks of feeding, the larval ectoderm shrinks and the developing ventral half of the little adult with its spines, tube feet and pedicellaria emerges (Czihak, 1971, Cameron and Hinegardner, 1974, Hinegardner, 1975). The remaining adult structures develop after metamorphosis (Hinegardner, 1975)

Lineage specific markers

Evidence discussed in Davidson (1986) suggests that the fate of some of the cells of the early embryo is determined by maternal cytoplasmic factors. The micromere lineage epitomizes this process of determination: even when cultured in isolation, micromeres form skeletal elements. Their fate seems to depend on maternal cytoplasmic determinants localized in the vegetal pole of the egg and inherited by micromeres during early cleavage. Classical transplantation experiments have shown that such determined cells have inductive interactions with their more plastic neighbors and influence their fate (reviewed in Czihak, 1971) Thus, the development of the sea

urchin embryo depends on determinants localized in the egg as well as cell-cell interactions. In the undisturbed embryo, cell lineages are established quite early (Cameron et al, 1987) although not irreversibly (Ettensohn and McClay, 1988)

By microinjecting fluoresceinated dextran into individual blastomeres at various stages of development, Cameron et al (1987) showed that embryonic tissues are clonal in origin. For example, the oral and aboral ectoderm lineages are specified as early as the 5th and 6th cleavages although expression of lineage specific genes cannot be detected until much later Oral ectoderm derives exclusively from animal cells whereas both animal and vegetal cells contribute to the aboral ectoderm

Lineage specific markers are useful tools to estimate the level of commitment of a cell to a specific differentiation pathway Much effort has been devoted to the identification of genes or gene products that are specific for certain embryonic tissues Cytoplasmic actin genes CyIIIa and b (Cox et al, 1986) and the Spec genes (Bruskin et al, 1981, 1982) are examples of genes expressed specifically in ectoderm cells.

Spec3

In an attempt to identify markers of ectoderm and mesoderm/endoderm differentiation, Bruskin et al (1981) isolated five cDNAs corresponding to mRNAs enriched in ectoderm fractions of the sea urchin S. purpuratus relative to endoderm/mesoderm fractions They refered to this set of cDNAs as Spec1, 2, 3, 4 and 5 Spec stands for S. purpuratus ectoderm.

The Spec1 and Spec2 genes code for small acidic calcium binding proteins related to the troponin C family (Carpenter et al, 1984)

Eldon et al (1987) presented evidence that Spec3 is involved in ectodermal ciliogenesis. Transcribed from a single gene, the Spec3 message is present at low level before fertilization and during the first 4 or 5 cleavages Then its level increases rapidly to reach a maximum at the blastula stage when it is 50 times more abundant than in unfertilized eggs. After a rapid decline to about 25% of its maximum by gastrula, it accumulates again at prism and pluteus stages. During this second phase of accumulation. the Spec3 message reaches about half the maximum level observed at the blastula stage

In situ hybridization of a Spec3 probe to sections of embryos at different stages showed that the Spec3 transcript is present in all cells of the very early blastula but eventually becomes limited to the ectodermal lineage (Eldon et al, 1987) In sections of mesenchyme blastulae, the probe did not detect the Spec3 transcript in the vegetal plate region or in the mesenchyme cells At the gastrula stage, grain density was high in the region of the presumptive ciliary band, but there was no signal in the presumptive oral ectoderm, the endoderm or the mesenchyme cells In plutei, the signal was very intense in the region of the ciliary band

Since the pattern of accumulation and of localization of the Spec3 message is very similar to that of tubulin and transcription from both types of genes increases in response to deciliation (Harlow and Nemer, 1987, Gong and Brandhorst, 1987), Eldon et al (1987) concluded that Spec3 is probably involved in ectodermal ciliogenesis

Eldon et al (1989) looked at the localization of the Spec3 protein by immunofluorescence staining of fixed embryos with an antiserum raised against a peptide corresponding to the predicted amino terminal region of the Spec3 protein. The pattern of staining at gastrula stage is striking (see Fig. 1 in the Results section) The embryo ectoderm is regularly spotted. A different plane of focus shows that ectodermal cilia are also stained.

The localization of the bright spots in the apical region of the cell at the basis of each cilium, along with evidence from drug treatments and immunogold labeling indicate that these structures correspond to Golgi complexes When Brandhorst (Eldon et al, 1989) treated embryos with colcemid, a microtubule disrupting agent, the pattern of staining with the Spec3 antiserum was modified. instead of a regular array of bright spots, ectodermal cells exhibit a fine punctate staining of the cytoplasm, analogous to the labeling of the Golgi complex in mammalian cells treated with microtubule depolymerizing agents (Lin and Queally, 1982, Lipsky and Pagano, 1985, Allan and Kreis, 1986) Thus, results from Brandhorst with colcemid are consistent with the localization of Spec3 in the Golgi apparatus which is held in place by microtubules Staining of the cilia is not altered by colcemid treatment When Decker labeled thin sections of embryos with colloidal gold (Eldon et al 1989), the only cytoplasmic structures that were consistently labeled were the Golgi cisternae Finally, when Brandhorst (Eldon et al, 1989) treated embryos with monensin, an inhibitor of Golgi mediated protein secretion, he observed swelling of the bright spots, again suggesting that Spec3 is localized in the Golgi complex

Sea urchin cilia

The first detectable morphogenetic event in the developing embryo is the growth of cilia at the blastula stage. Depending on urchin species, cilia are present on all cells in the blastula or on all cells except the micromeres (Masuda, 1979) Collectively, they beat in a wave-like progression called metachronal movement, propelling the embryo in sea water Metachronism regulates the flow of water around the embryo (Sleigh and Aiello, 1971)

At the animal pole, the apical tuft cilia are immotile and therefore termed stereocilia Trypsin or concanavalin A treatment at the blastula stage causes animalization of the embryo, a condition characterized by the elongation of cilia all around the blastula Other animalizing agents, such as zinc or thiocyanate have no effect so late in development. Upon trypsin treatment, short and motile cilia become longer and unable to move (Burns, 1979, Riederer-Henderson and Rosenbaum, 1979). Curiously, if such animalized embryos are deciliated with hypertonic sea water and allowed to regenerate their cilia in the absence of trypsin, they regenerate long and immotile cilia (Riederer-Henderson and Rosenbaum, 1979) Trypsin treatment thus appears to change irreversibly the program that determines the length of cilia Evidence from Burns (1973, 1979) and Stephens (1977b) in sea urchin and other systems (reviewed by Lefebvre and Rosenbaum, 1986) indicates that the size of the ciliary precursor pool is not a factor in determining the length of cilia or flagella.

In sea urchin embryos, a large pool of ciliary proteins (Raff, 1975, Bibring and Baxandall, 1981) and mRNAs (Alexandraki and

Ruderman, 1985) is present before ciliogenesis. Ciliary regeneration can take place in the presence of inhibitors of RNA synthesis (actinomycin D) and protein synthesis (puromycin) (Auclair and Siegel, 1966) However, if protein synthesis is inhibited with pactamycin just before regeneration, cilia do not grow (Child and Apter, 1969), suggesting that there is a time window when critical components for ciliary assembly are synthesized

Stephens (1972, 1977a) analyzed the timing of ciliary protein synthesis by labeling the proteins of developing embryos with pulses of radioactive precursors at various time points during the course of ciliogenesis and by measuring the incorporation of label in ciliary proteins He identified four different types of ciliary proteins relative to the timing of their synthesis: proteins that pre-exist in the unfertilized egg, proteins that are synthesized continuously before ciliogenesis, proteins that appear to decrease with time and proteins that are synthesized *de novo* during ciliogenesis

In another series of experiments, Stephens (1977b) deciliated embryos and labeled ciliary proteins during regeneration. He deciliated embryos again and allowed them to regenerate their cilia in the absence of labeled precursors In general, there is a decrease in the intensity of labeling of ciliary proteins after the second regeneration. A subset of proteins is labeled with a high specific activity during the pulse and their labeling declines abruptly during the second regeneration, as if most of the proteins synthesized during the pulse were incorporated in the first regeneration and only newly synthesized, unlabeled proteins contributed to the second regeneration This subset includes a component of the protofilament

ribbon at the junction of the A and B tubules, probably tektins, the protein nexin; and other unidentified components of the A and B tubules Stephens (1977b) proposes that these proteins are synthesized in limiting amounts during a specific brief period after deciliation and have a critical role in initiating cilia formation Other proteins such as tubulin and dynein are more abundant in cilia harvested after the cold regeneration than in cilia grown during the pulse. Stephens (1977b) suggests that this delayed utilization could be due to a lag in synthesis or processing or to compartmentalization after synthesis

Structure of the ciliary and flagellar axoneme

Cilia and flagella are filamentous motile organelles that project out of the cell body Their structure is very similar in all eukaryotic organisms They have in common many proteins and a characteristic structure the axoneme Flagella are usually 2 to 3 times longer than cilia and they beat with a continuous, sinusoidal pattern whereas ciliary movement is biphasic with a power stroke and a recovery stroke (Stephens, 1977c) analogous to breast stroke swimming. Usually, numerous cilia cover large epithelial surfaces whereas flagella are present in small numbers on unicellular organisms. However, in practice there is a continuum between the two extremes, in terms of length and motion patterns (Sleigh, 1974) Cavalier-Smith (1982, 1986) argues that there is no rational basis for distinguishing cilia and flagella He uses the term cilia form all eukaryotic ciliary and flagellar appendages and reserves the term flagella for prokaryotes

The axoneme extends from a cytoplasmic basal body and is enclosed in a membrane that is continuous with the plasma membrane This organelle is a cylinder of nine doublet microtubules surrounding a pair of single microtubules, referred to as the 9+2 structure. Each doublet is made from a whole tubule, the A-tubule, to which is apposed a C-shaped incomplete tubule, the B-subfiber. The A-tubules and the tubules from the central pair each contain 13 protofilaments made of α- and β-tubulin heterodimers, whereas each B-subfiber is made of 10 or 11 protofilaments and spans 5 protofilaments from the A-tubule (Linck and Langevin, 1981). Three protofilaments that make the junction between the two elements of the doublet are called junctional protofilaments or ribbons. They contain the tektins, proteins of molecular weights of 47, 51 and 55 kD, as well as tubulin (Linck et al, 1985, Linck and Stephens, 1987). Linck (1982) proposes that tektins are related to intermediate filaments

Two projections are attached to the A-tubule They are the inner and outer dynein arms (described in sea urchin flagella by Tang et al, 1982 and Ogawa and Gibbons, 1976) that generate ATP-dependant movement by forming cross-bridges with the B-subfiber of adjacent doublets, causing them to slide against each other The name dynein is derived from the word dyne, a unit of force (reviewed in Gibbons, 1981)

Stephens (1970a) proposes that nexin (from the latin nexus: link) is a protein that keeps outer doublets together and maintains them in a ring shape. A set of radial spokes connects the central pair of microtubules to each A-tubule of the outer doublets Each spoke consists of a spoke head on the end facing the central pair and

a stalk (Hopkins, 1970). The nexin ring and the radial spokes protect the axoneme from dismantling by stress or dynein mediated sliding. Two projections extend from each of the two central microtubules. Collectively, they are called the central sheath (Warner and Satir, 1974). There is also a pair of cross bridges that joins the two central tubules together (Gibbons, 1981).

Mastigonemes or flagellar hairs are structures that extend from flagellar outer doublets through the flagellar membrane (Bouck, G.B., 1971, Witman et al., 1972). They are found in algae, fungi, zooflagellates, actinopods and sponges (Bouck, 1972, Cavalier-Smith, 1982, 1986). Their assembly begins in the nuclear envelope and they transit in the Golgi before being added to the proximal end of the flagellum (Bouck, 1972, Domnas et al, 1986). After extraction of the flagellar membrane, mastigonemes remain attached to flagellar outer doublet microtubules (Bouck and Green, 1976). Purified and electrophoresed mastigonemes from Chlamydomonas reinhardtii yield a single polypeptide. This protein has an apparent molecular weight of 170 kD and has a positive reaction to carbohydrate staining (Witman et al, 1972). Kawano and Bouck (1983) purified 5 different glycoproteins from Ochromonas mastigonemes .

Flagellar structure and motility have been studied extensively in the unicellular alga Chlamydomonas reinhardtii because of the availability of flagellar mutants (reviewed in Luck, 1984, Huang, 1986; Lefebvre and Rosenbaum, 1986). Of the 150 axonemal polypeptides that can be identified on two-dimensional gels, about 75 can be associated to specific structures of the axoneme by mutational

dissection (for example, Piperno et al, 1977, Piperno et al, 1981, Remillard and Witman, 1982).

There is an inherent structural polarity in Chlamydomonas flagellar axonemes. Hoops and Witman (1983) describe 3 markers of this polarity. There is a pointed "beak-like" structure in the lumen of the B-subfiber of doublets 1, 5 and 6; doublet 1 does not have an outer dynein arm; and there is a 2 part bridge (different from dynein arms) between the A-tubule of doublet 1 and the B-subfiber of doublet 2 (in most organisms, however, the bridge is between doublets 5 and 6). Interestingly, the doublet with no outer arm always faces the other flagellum.

Chlamydomonas has two flagella that beat with the ciliary pattern when the cell moves forward and with the flagellar pattern when it moves backward. The change in swimming pattern is regulated by the level of intracellular Ca^{+2} and is a property of the axoneme itself (Nakamura, 1981).

Many mutants isolated by Nakamura (1979, 1981) and Segal et al (1984) are unable to move with the ciliary pattern and can only perform the symmetric flagellar bending pattern characteristic of backward movement All these mutants have in common that they are deficient in the "beak-like" projections normally found on the B-subfibers of doublets 1, 5 and 6. A set of six polypeptides of molecular weights ranging from 30 to 245 kD are commonly missing in all the mutants. Two of these polypeptides are not phosphorylated properly in some of the mutants (Segal et al, 1984).

Reassembly of microtubules

Cytoplasmic microtubules can be depolymerized by cold or colchicine treatment. They repolymerize when the temperature is raised. When cycles of assembly-disassembly are performed with brain extracts, specific high molecular weight proteins copurify with tubulin and are essential for microtubule reassembly when the tubulin concentration is below a critical point (reviewed by Olmsted, 1986) These microtubule associated proteins (MAPs) have been the focus of much attention over the past few years.

As opposed to cytoplasmic microtubules, ciliary and flagellar microtubules are resistant to depolymerization by cold or colchicine Kuriyama (1976) and Farrell and Wilson (1978) used sonication to depolymerize doublet microtubules.

The A-tubule and B-subfiber of the ciliary and flagellar microtubules are solubilized differentially at high temperatures the B-subfiber is depolymerized more rapidly when exposed to heat (Stephens, 1970b). Linck and Langevin (1981) used this property of outer doublets to purify tubulin from the B-subfiber of sea urchin sperm flagella by successive rounds of polymerization and depolymerization.

When solubilized by heat treatment and allowed to repolymerize, B-subfibers of flagellar outer doublets reassemble into singlet microtubules containing 12 to 15 protofilaments, most frequently 13 protofilaments (Linck and Langevin, 1981) Interestingly, even if the tubulin is over 95% pure after the second round of polymerization, it is able to reassemble at concentrations much lower than what is usually needed for brain tubulin reassembly. Actually, the critical

concentration for reassembly of B-subfiber tubulin is similar to that of brain tubulin in the presence of MAPs. Linck and Langevin (1981) suggest that a "potent nucleating protein", a MAP, might be present in flagellar extracts as an undetected contaminant.

Microtubules reconstituted from sonicated outer doublets become sensitive to cold and colchicine, indicating that the stability of the outer doublet is a property of its structural organization rather than of tubulin itself (Kuriyama, 1976; Farrell and Wilson, 1978).

In practice, the term MAP has evolved to describe any protein that interacts, even transiently, with microtubules (Olmsted, 1986). Under such a vague definition, many more proteins might qualify as MAPs.

MATERIALS AND METHODS

MATERIALS

Supplies

Strongylocentrotus purpuratus and Lytechinus pictus sea urchins were purchased from Marinus, Long Beach, California. L-azetidine-2-carboxylic acid, monensin, puromycin and tunicamycin were purchased from Sigma Chemical Company, trypsin from Boehringer Mannheim. All drugs were dissolved in distilled water except monensin which was made 5mM in 75% methanol. Anti-tubulin antiserum (whole serum), raised in rabbits against sea urchin tubulins, was purchased from Polysciences Inc Fluorescein conjugated goat anti-rabbit antibody was purchased from Cappell (Organon Teknika Corporation), ^{125}I labeled protein A from ICN Biomedicals Inc. Proteins were transfered onto Immobilon-P (PVDF) membrane obtained from Millipore Corporation Silver staining of gels was done using a kit from Bio-Rad Laboratories Molecular weight markers were SDS-6H and SDS-7 mixtures from Sigma which contain the following proteins. myosin (205 kD), β-galactosidase (116 kD), phosphorylase B (97 4 kD), bovine albumin (66 kD), egg albumin (45 kD), glyceraldehyde 3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 1 kD) and α-lactalbumin (14 2 kD)

Spec3 antiserum

The Spec3 antiserum and the peptide used to generate it were donated by the laboratory of W H Klein. The antiserum was raised in

rabbit against a synthetic peptide corresponding to the deduced first 14 amino terminal amino acid residues of the Spec3 protein, and conjugated to keyhole limpet hemocyanin. An IgG fraction was isolated by passage through a DEAE Affi-Gel Blue column (Bio-Rad Laboratories) and antibodies against hemocyanin were removed by affinity chromatography (Eldon et al, 1989)

METHODS

Culture of embryos

Gametes were obtained by intracoelomic injection of 0.5M KCl, 1 ml for S. purpuratus, 0.1 ml for L. pictus, and fertilized in artificial sea water (recipe from the Marine Biological Laboratory) Embryos were cultured at a concentration of less than 3000/ml at a temperature of 14 to 16°C. At 16°C, S. purpuratus embryos hatch in about 12 hours, become mesenchyme blastulae in 18 hours, begin to gastrulate after 24 hours, reach the prism stage in 48 hours and the pluteus stage in 72 hours

Preparation of cilia

Cilia preparation was carried out using a modification of the method of Merlino et al (1978). 0 12 volume of 4.45 M NaCl was added to a suspension of ciliated embryos and the mixture was swirled for 2 minutes Embryos were then pelleted in a clinical centrifuge, the supernatant was transfered to clean tubes containing a cotton wad at the bottom and centrifuged again to remove all remaining embryos (Stephens, 1986). The supernatant containing cilia was transfered to

Corex tubes and cilia were pelleted by centrifugation at 5000g for 30 minutes. For cilia regeneration, deciliated embryos were resuspended in artificial sea water and cultured normally.

Preparation of sperm flagella

Sperm tails were prepared according to Stephens (1986). Briefly, debris were removed from a sperm suspension by passage through several layers of cheesecloth. Spermatozoa were decapitated by homogeneization with about 10 strokes of a glass Dounce homogeneizer and centrifuged at 1000g for 5 minutes to remove intact sperm and heads. Flagella were then collected by centrifugation at 10,000g for 5 minutes.

Removal of the fertilization envelope

Eggs were fertilized in the presence of 3 mM para-amino-benzoic acid to prevent hardening of the fertilization envelope, stirred for 30 minutes and then passed through a 55 micron nylon membrane (Nitex) to remove the envelope (Dr Fred Wilt's notes, Embryology course, MBL, Woods Hole, Massachussets). Zygotes were allowed to settle, water was removed and replaced by artificial sea water. Embryos were then cultured as described above

Immunofluorescence staining of embryos and cilia

Packed embryos were fixed in 90% methanol, 10 mM EDTA for 5 to 10 minutes at -20°C (Harris, 1986), rehydrated twice in PBS (0.01 M each of Na_2HPO_4 and NaH_2PO_4, pH 7 4, 0.15 M NaCl) for 10 minutes or more. Embryos were allowed to settle between each change of solution

Non-specific binding sites were then blocked by addition of a blocking solution containing 3% bovine serum albumin, 10 % fetal calf serum in PBS for at least 1 hour. Fixed embryos were incubated at room temperature for 1 hour with the primary antiserum at a concentration of 50 μg/ml in the blocking solution, followed by 3 washes in PBS (10 minutes each). In certain cases, the Spec3 primary antiserum was preincubated with the N-terminal peptide used to generate it. The primary antiserum was used at a concentration of 10μg/ml; the peptide at 100μg/ml.

Embryos were then incubated for 1 hour with the fluorescein labeled goat anti-rabbit secondary antibody diluted 30 fold in blocking solution and washed 3 times for 10 minutes in PBS. To reduce photobleaching, samples were diluted 1:1 with 2 mg/ml paraphenylenediamine in PBS (Valnes and Brandzaeg, 1985) before mounting under a coverslip. Samples were observed and photographed with a Leitz Orthoplan fluorescence microscope, with excitation at about 445 nm and emission at about 520 nm. Nuclei were sometimes counterstained with 0.0001% ethidium bromide in PBS prior to observation. Staining of isolated cilia and axonemes was done similarly except that the preparation was centrifuged in an Eppendorf microcentrifuge between each change of solution. Axonemes were prepared by removing membranes with Triton X-100 (see below, and Stephens, 1986, for protocol).

Fractionation of cilia

Several fractionation protocols were applied to isolated cilia All steps involved extraction with a solubilizing agent and

sedimentation of the residue by centrifugation. All soluble fractions were precipitated in 80% acetone. Membrane proteins were solubilized by Triton X-100 extraction (Stephens, 1986). Cilia were washed in 30 mM Tris-HCl, 3 mM $MgCl_2$, 0.1 mM EDTA, pH 8 (TME), centrifuged in a micro-centrifuge and resuspended in TME containing 1% Triton X-100. The mixture was left on ice for 30 minutes and the axonemal fraction was pelleted in a micro-centrifuge and washed again in TME.

Alternatively, membrane proteins were separated into integral membrane proteins and other membrane proteins by differential solubilization into Triton X-114 (Dentler, 1988). Cilia were washed in cold PEMKS (50 mM Pipes, pH 7.1 with KOH, 3 mM $MgSO_4$, 0.1 mM EGTA, 10 mM potassium acetate, 1 mM EDTA, 250 mM sucrose). They were then suspended in 1% ice-cold Triton X-114 in PEMKS and incubated on ice for 20 minutes with frequent mixing. Axonemes were pelleted by centrifugation in a micro-centrifuge, the supernatant was carefully removed, placed in a 1 5 ml centrifuge tube and warmed in a 30°C water bath for 5 minutes until the solution became turbid The sample was centrifuged for 10 minutes at 1800g at room temperature The clear aqueous phase was removed from the cloudy detergent phase and each fraction was put on ice for precipitation in 80% acetone The axonemal pellet was washed again in PEMKS.

The remaining axonemes were subjected to high salt extraction according to Bell et al (1982) or Stephens (1986). These extraction methods solubilize dyneins in sperm flagella but in cilia, half of the dyneins and all the B-subfibers are released (Stephens, 1986) The method from Bell et al involves incubating axonemes for 15 minutes on ice in 0.6 M NaCl, 5 mM Imidazole-HCl, pH 7, 4 mM $MgSO_4$,

1 mM $CaCl_2$, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM dithiothreitol followed by centrifugation at 12,000g for 15 minutes Stephens uses 0 6 M KCl, 10 mM Tris, pH 8 and 0.1 mM dithiothreitol for 30 minutes at 4 °C.

The residual pellet was subjected to a Sarkosyl extraction, (Stephens, 1978) which solubilizes all but the junctional protofilament ribbons and associated proteins. In this case, the pellet was suspended in 0.5% Sarkosyl in 1 mM Tris-HCl, pH 8, 0.1 mM EDTA and 0 01% 2-mercaptoethanol on ice for 30 to 60 minutes The insoluble fraction was pelleted by centrifugation at 60000 rpm in a TLA-100.3 rotor in a Beckman TL-100 ultracentrifuge, for 30 minutes at 4 °C.

On other occasions, the high-salt insoluble pellet was subjected to a Sarkosyl-urea extraction which for sperm flagella solubilizes everything but tektins (Linck et al, 1985). The pellet was suspended in 2.5 M urea. 0.5% sarkosyl in 10 mM Tris-HCl, 1 mM EDTA, pH 8 for 2 hours on ice and then centrifuged for 2 hours at 70000 rpm in a Beckman TL-100 ultracentrifuge.

Reduction and alkylation of proteins

Ciliary proteins were diluted in water at a concentration of 1 mg/ml. An equal volume of 1.5% SDS, 20 mM DTT was added and the mixture was incubated at 85°C for 10 minutes. Fresh N-ethyl-maleimide was added to a final concentration of 0 04 M and incubated on ice for 1 hour Then, 1/5 of the total volume of 50% glycerol, 0 5% 2-mercaptoethanol, 0 001% bromophenol blue was added and samples were run on SDS gels (Harlow and Lane, 1988).

Protein electrophoresis and immunoblotting

Proteins samples were dissolved in SDS sample buffer (Laemmli, 1970) and boiled for 5 minutes. Discontinuous SDS polyacrylamide gel electrophoresis was done according to Laemmli (1970). When necessary, the protein concentration was estimated by the Coomassie staining method of Esen (1978). Gels were stained using the silver staining kit from Bio-Rad

Gels were electroblotted onto Immobilon-P membranes in cold 25 mM Tris-HCl, pH 8 3, 192 mM glycine, 15% methanol (Towbin et al, 1979)and 0.5% SDS at about 300 mA overnight and then blocked for at least 6 hours in Blotto (50 mM Tris, pH 7 5, 0 9% NaCl, 0 05% Tween-20, 3% Carnation non-fat dry milk, 0 05% NaN_3, filtered through Whatman #1 paper) Blots were incubated for 2 hours with the primary antiserum diluted in Blotto (10-20 μg/ml), rinsed 3 times with Blotto, washed twice in Blotto for 20 minutes and then washed overnight in Blotto. In some experiments, the primary antiserum was preincubated with the N-terminal peptide used to generate it at a final concentration of 100μg/ml Blots were incubated with iodinated protein A (1:1000 in Blotto) for 2 hours and washed as described above. Blots were then exposed to pre-flashed X-ray films at -80°C with intensifying screens

RESULTS.

DISTRIBUTION OF THE SPEC3 ANTIGEN DURING EMBRYOGENESIS

Gastrula and prism

Indirect immunofluorescence staining of gastrula and prism embryos with the Spec3 antiserum revealed a striking pattern of distribution for this antigen. At low magnification, it appeared as a regular array of bright spots on the entire surface of the embryo (Fig 1a) At higher magnification, these spots were resolved into rings or comma-shaped structures (Fig. 2a) As shown by ethidium bromide counterstaining, each spot was closely apposed to a nucleus (Fig 3) Cilia seemed to originate from these structures and stained as strings of beads (Fig. 2b). There was no staining with the pre-immune serum (Fig 1c)

The localization of the bright spots in the apical region of the cell at the basis of each cilium, along with evidence from colcemid and monensin treatments and immunogold labeling indicate that these structures correspond to Golgi complexes (see literature review on Spec3).

Figure 1. **Distribution of Spec3 in gastrula embryos.**

a) Immunofluorescence staining of a gastrula with the Spec3 antiserum The diameter of the embryo is about 100μm.

b) Bright field image of (a).

c) Gastrula stained with the preimmune serum

d) Bright field image of (c)

(courtesy of E D. Eldon)

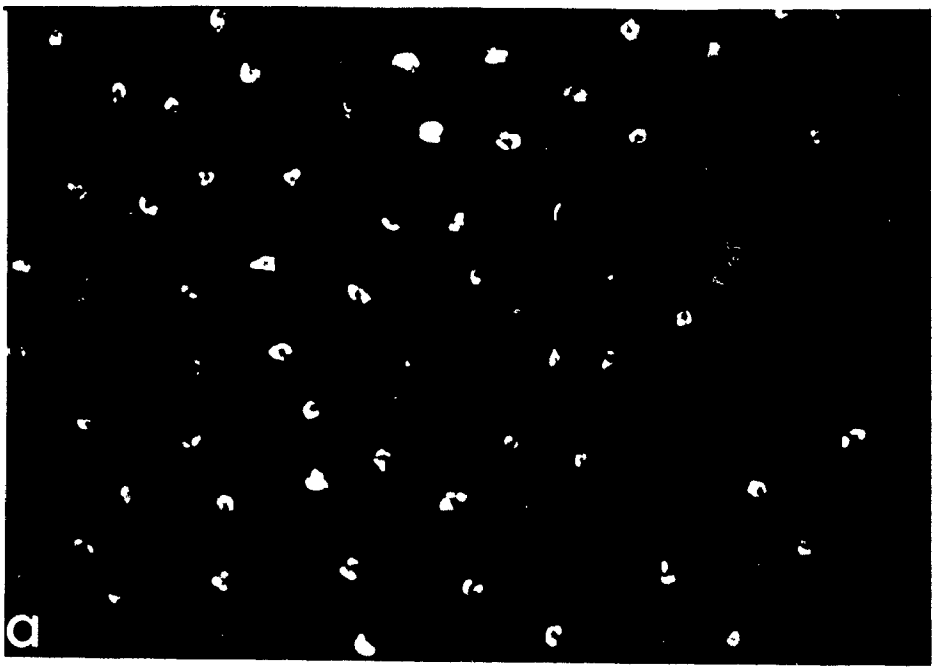
a
c

Figure 2. **Distribution of Spec3 in gastrula embryos.**

Immunofluorescence staining of a gastrula with the Spec3 antiserum.

a) Focus was just below the ectodermal surface of the embryo, showing apical structures. (x790)

b) Focus was on cell surface, showing cilia. (x790)


a


b

Figure 3. **Staining of Spec3 and nuclei.**

A prism embryo was stained by immunofluorescence with the Spec3 antiserum. Nuclei were counterstained with ethidium bromide. Nu: nucleus; Ci: cilium; Go: Golgi apparatus.

Pluteus

The aboral ectoderm region of pluteus larvae stained with a pattern similar to gastrula or prism stages Fig. 4a and b show beaded cilia attached to Golgi at their proximal end. At that stage, the Golgi complexes were smaller than at the earlier stages

The pluteus larva is characterized by the appearance of a ciliary band surrounding the oral surface This band stained very brightly with the Spec3 antiserum because of the presence of several rows of closely spaced cilia (Fig. 4a). In most preparations, there was no obvious staining of the Golgi in the cells of the ciliary band and the oral area (see also section on deciliation and monensin treatment)

In some cases, there was a double row of stained circular structures at the base of the ciliary band (Fig 4c) These structures were clearly different from Golgi complexes. their diameter was much larger, they did not stain as brightly and their edges were not as sharply defined When the same preparations were stained for tubulin, the pattern was similar (Fig 4d) For a Japanese species of urchins, Nakajima (1986a) described coiled cilia at the surface of the preoral area of the pluteus Although the structures that I observed were not in the preoral area but in the ciliary band, their appearance and the presence of tubulin suggest that they are coiled cilia

The mouth of the pluteus larva is surrounded by cilia that reacted with the Spec3 antiserum (Fig 4e) When stained, these cilia were shorter than those from the aboral ectoderm and the ciliary band and their distal end was round and club-shaped. Thus, as opposed to

other ectoderm specific genes such as Spec1 or the actin genes CyIIIa and CyIIIb, the expression of Spec3 is not limited to the aboral ectoderm

The presence of the Spec3 antigen in the oral area is also contradictory to results of in situ hybridizations obtained by Eldon et al (1987) where Spec3 transcripts were not detected in the oral ectoderm. Since the Golgi of oral cells do not seem to contain any of the Spec3 protein, the mass of Spec3 protein per cell is probably less than in the aboral ectoderm. The Spec3 message in the oral ectoderm may not be abundant enough to be detected by in situ hybridization.

Campos and Mann (1988) described modified cilia in larvae of bivalves. Their scanning electron micrographs illustrate cilia with enlarged distal ends that ressemble the stained circumoral cilia of Fig. 4e They suggest that these "discocilia" could be involved in chemosensation or in enhancing movement. Modified cilia around the mouth of urchin larvae may have a function in screening and directing food particles before ingestion.

Figure 4. **Immunofluorescence staining of pluteus embryos.**

Panels a, b, c and e are labeled with the Spec3 antiserum, panel d with an antiserum against tubulin.

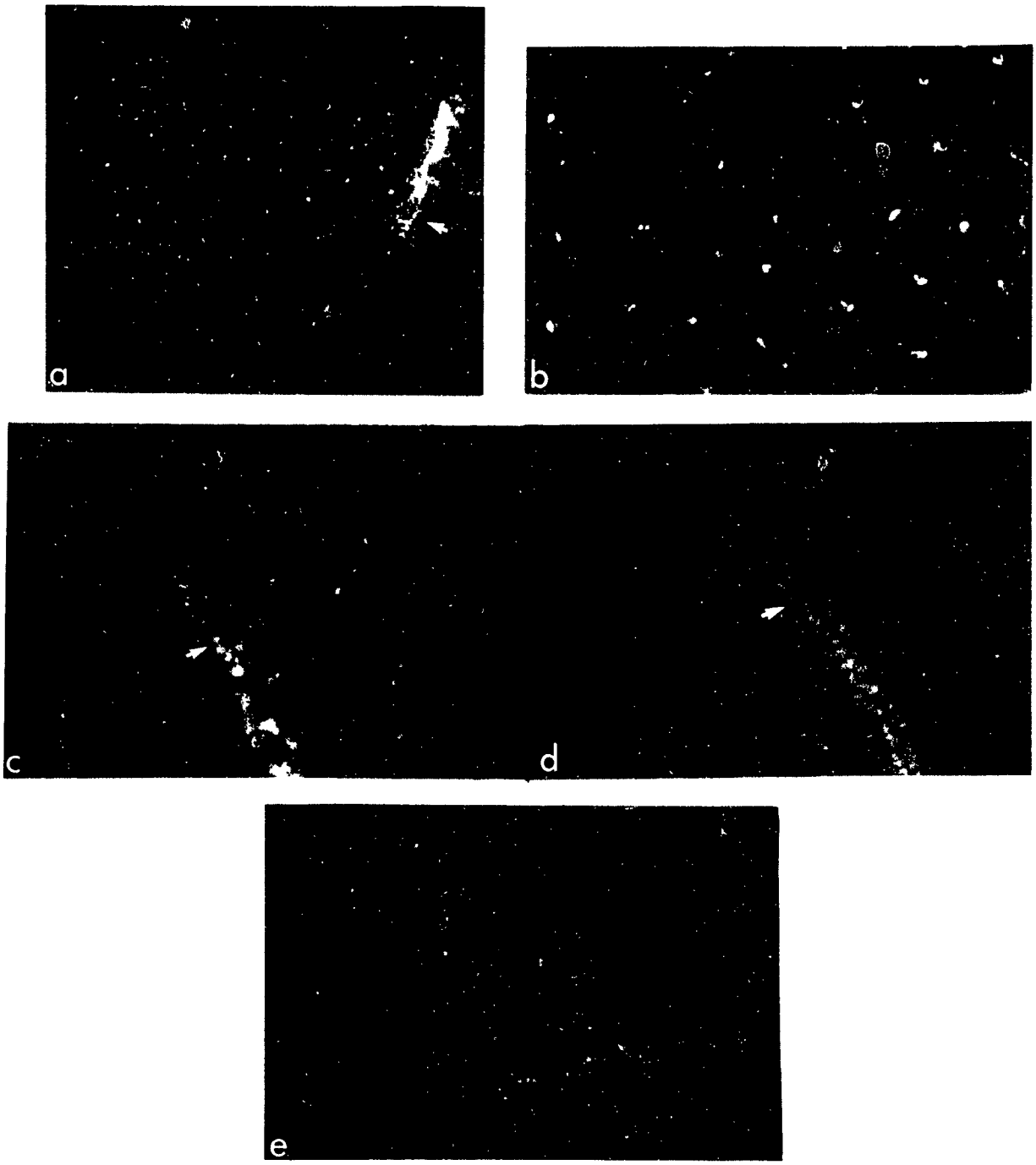
a) Pluteus larva stained with the Spec3 antiserum Arrow points to the ciliary band between oral and aboral surfaces.

b) Higher magnification (x810) of the aboral surface showing the beaded pattern of cilia and the associated apical structure at the basis of each cilium

c) Double row of round structures at the base of the ciliary band (x500)

d) Structure similar to that in (c) stained with an antiserum against tubulin (x500)

e) Staining of rows of cilia around the mouth of a pluteus Arrow points to the bulbous distal end of a cilium (x500)


a
b
c
d
e

No staining was ever observed on cells other than ectodermal cells. Cells of the gut are ciliated but endodermal cilia did not react with the Spec3 antiserum, indicating that Spec3 is specific to ectodermal cilia.

Blastula

Since the Spec3 protein is strongly associated with ectodermal cilia, it was interesting to look at its distribution at the blastula stage, when cilia first appear. The fertilization envelope was removed after fertilization to allow immunostaining of embryos before hatching. Normally, due to ciliary movement, blastulae start to spin inside their envelope before they hatch When the envelope was removed, they swam in sea water but their movement was disoriented compared to mesenchyme blastulae which tended to have a spiral movement toward one direction, animal half first

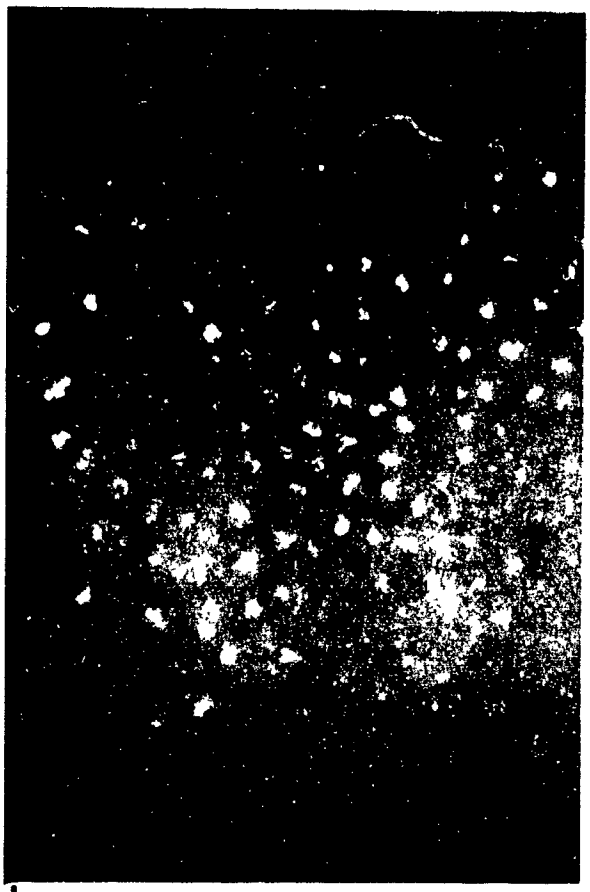
When embryos were stained before they began to move, the fluorescence was distributed throughout each cell in a bright punctate pattern (Fig. 5a) which merged into larger Golgi complexes when embryos started to move (Fig 5b) Staining of the Golgi did not appear simultaneously in all cells (Fig 5c) Before hatching of controls, there was no visible staining of the cilia

Figure 5 **Distribution of Spec3 in blastula embryos before hatching.**

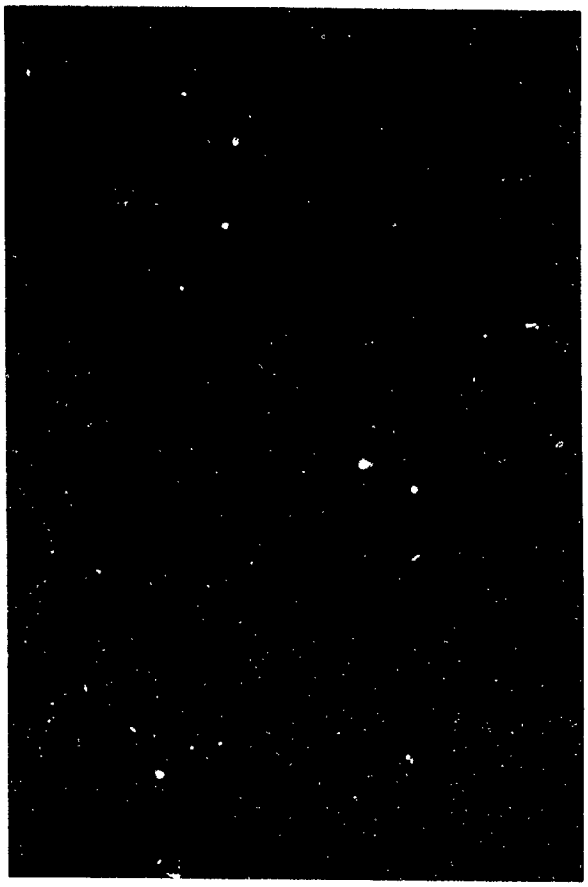
a) Immunofluorescence staining of a young blastula before any movement was visible. (x400)

b) and c) Blastula were stained when they started moving but before hatching (x630)

a



b



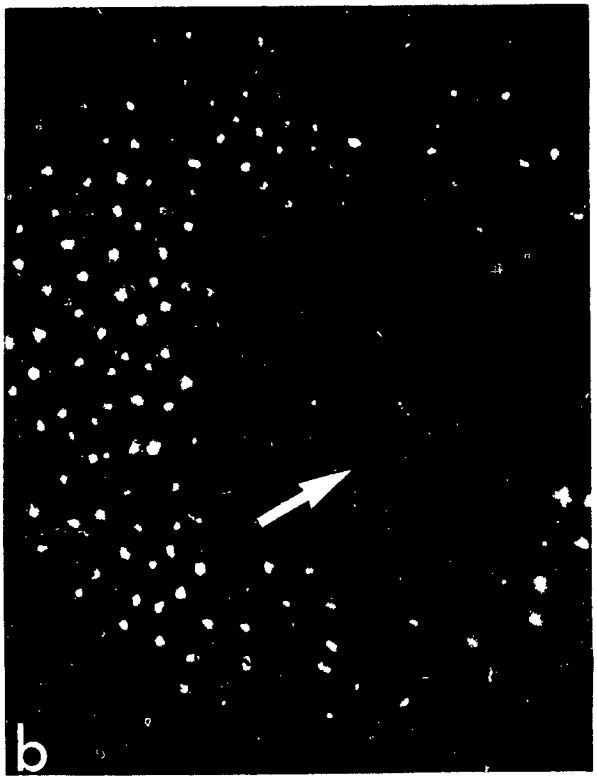
c

Figure 6 **Distribution of Spec3 in hatching blastula and mesenchyme blastula.**

a) Immunofluorescence staining of a hatching blastula Some cilia are stained (arrow) (x630)

b) and c) Staining of the same mesenchyme blastula, two different planes of focus (x630). In (b), focus is on apical structures, in (c), on cilia. Arrows point to the vegetal plate which does not stain with Spec3


a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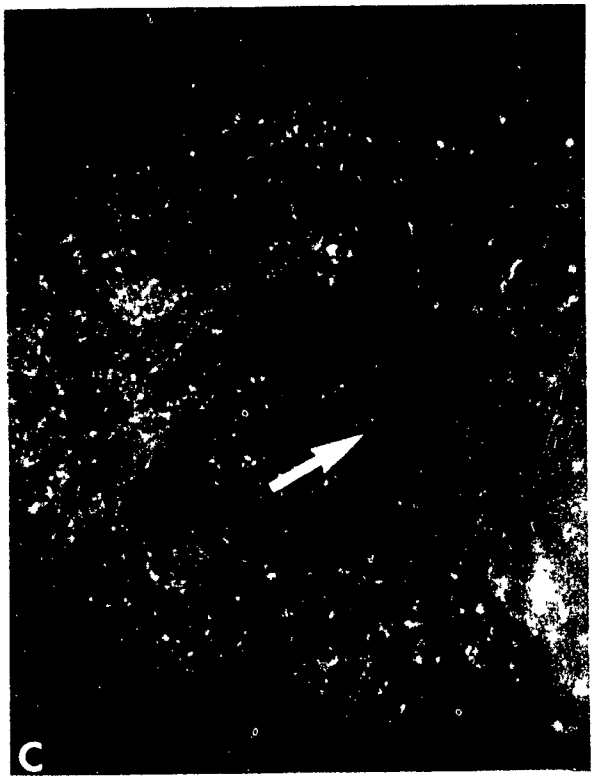

c

In hatching blastulae, there was no unique and uniform pattern of staining. Depending on cells and embryos, Spec3 was present in a fine punctate pattern or in larger Golgi complexes and sometimes, a few cilia per embryo were stained (Fig. 6a shows the extreme case of an embryo with many stained cilia). It seems that the organization of Spec3 into the Golgi and cilia is a process that takes place over several hours.

At the mesenchyme blastula stage, most ectodermal cells exhibited a bright Golgi and a beaded cilium (Fig 6b,c). However, cells of the vegetal plate that would later invaginate to form the endoderm and the secondary mesenchyme cells did not show any staining with the Spec3 antiserum (Fig. 6b,c).

The appearance of Spec3 on cilia is coincident with a transition in the pattern of movement of embryos. the erratic movement of pre-hatching blastulae is gradually replaced by a directional motion that is obvious by the mesenchyme blastula stage Whether Spec3 is involved in this transition is still to be determined.

Other stages

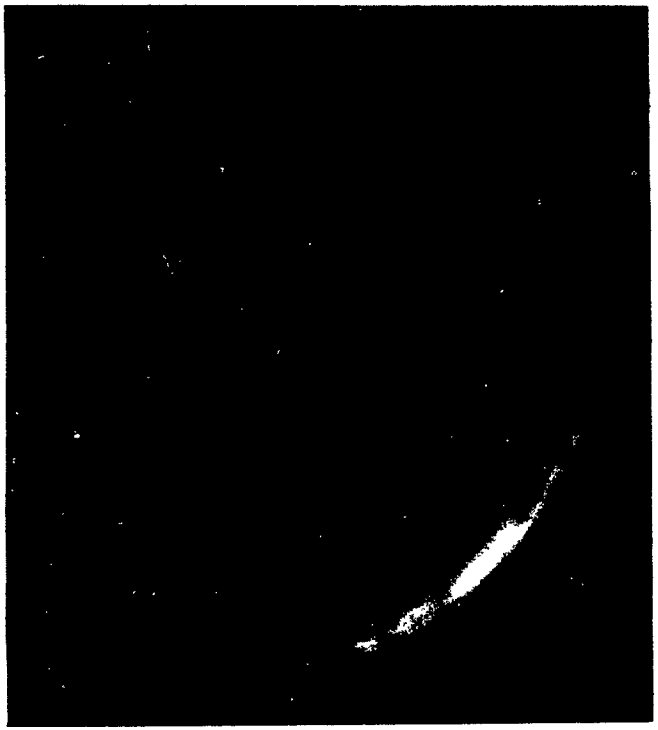
At the 16 cell stage, staining with the Spec3 antiserum was diffuse and there was a set of bright spots in the middle of each cell (Fig. 7). The overall intensity of staining was comparable to what was seen in blastulae although the pattern of staining was more diffuse, except for the sets of bright spots which were larger than each of the small spots seen in blastulae Since the level of staining with the Spec3 antibody in unfertilized eggs is much lower

than in blastula (Eldon, 1988), accumulation of the Spec3 protein must have started by the 16 cell stage although the message is still not abundant at that stage (Eldon et al, 1987). Alternatively, the increase in staining that I observed at the 16 cell stage could be the result of a change in the accessibility of the antigen to the antiserum

Since most of the larval ectoderm and ciliary band are discarded at metamorphosis (Davidson, 1986), the Spec3 protein that was synthesized during embryonic and larval development should disappear at metamorphosis I wanted to establish if the Spec3 antigen is present in metamorphosing larvae The only structure of the metamorphosing larva that stained with the Spec3 antiserum was the distal end of the tube foot (Fig. 8) Since most of the adult rudiment originates from mesenchyme progenitor cells that do not express Spec3 during embryogenesis, the presence of the antigen on the tube foot must result from new synthesis from the Spec3 gene or another gene homologous to Spec3. It implies that synthesis from that gene must be activated in a new set of cells. Alternatively, the tip of the tube foot could originate from the small region of the larval ectoderm that is involved in the formation of the adult rudiment (Hinegardner,1975)

Figure 7 **Distribution of Spec3 in 16 cell stage embryos.** (×520)

Figure 8 **Distribution of Spec3 in a metamorphosing larva.** The Spec3 antiserum stains the distal end of the tube foot.



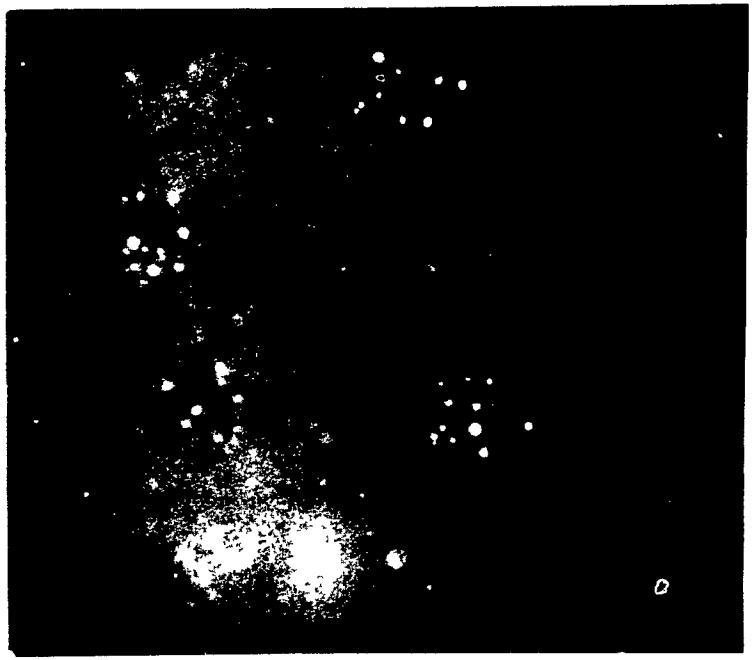
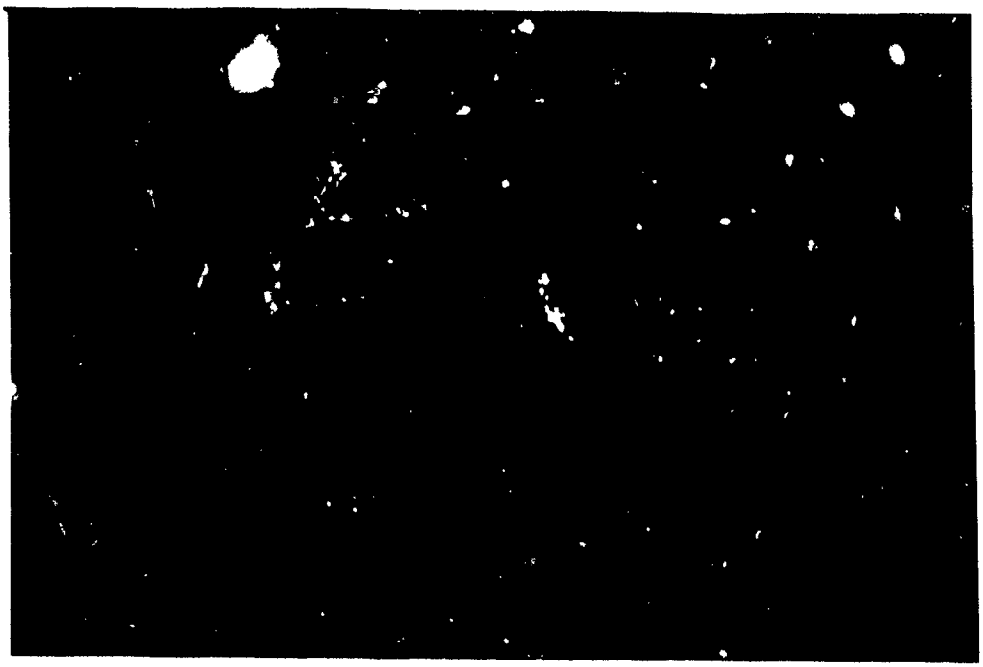


Figure 9. **Trypsin treatment of embryos changes the pattern of staining of Spec3 on cilia.**

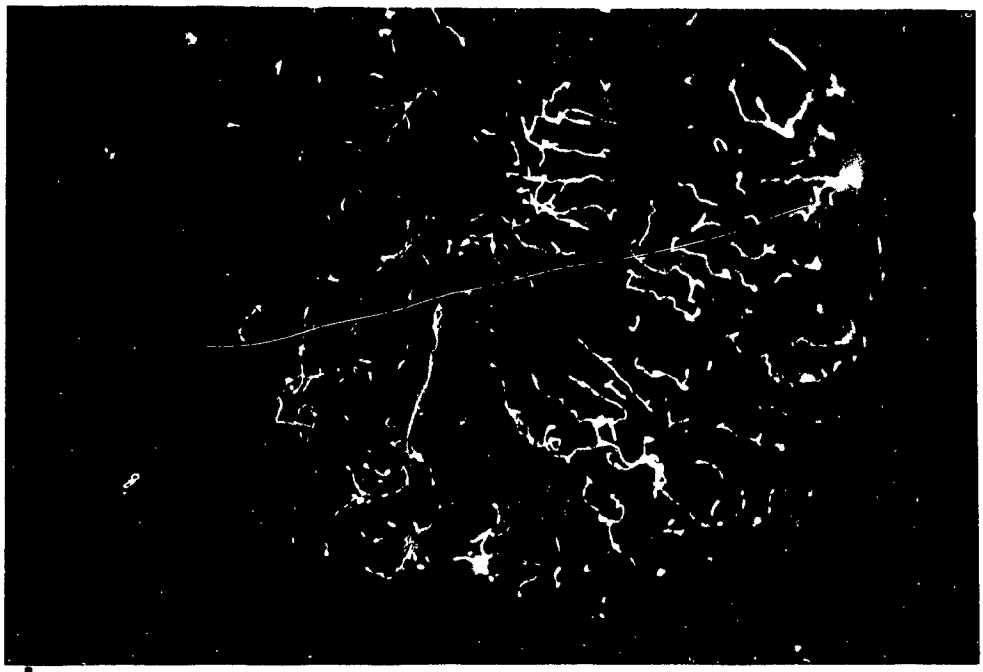
Analysis by immunofluorescence staining.

a) Control gastrula Cilia stain with a beaded pattern. (x780)

b) Embryo treated with trypsin for 1 minute. Cilia are brightly stained with a smooth pattern. (x780) Photography of (a) and (b) was done under the same exposure conditions.



a



b

Figure 10 **Distribution of Spec3 on isolated cilia and axonemes.**
a)Whole embryos were stained by immunofluorescence with the Spec3 antiserum and cilia were detached during the staining procedure Cilia exhibit the beaded pattern. (x700)
b) Embryos were deciliated and harvested cilia were stained with the Spec3 antiserum. The beaded pattern was lost and staining was less intense. (x700)
c) Cilia were demembranated before staining and axonemes were processed as in (b) The staining pattern is similar to (b). (x700)


a


b


c

EFFECT OF TRYPSIN

The Spec3 sequence contains five sites for trypsin cleavage (residues 55, 93, 94, 99, 145) (Eldon et al, 1987). If at least the 55 amino terminal residues of the protein were protruding out of the ciliary membrane, this region of the protein would be susceptible to trypsin cleavage in living embryos. Staining of cilia with the Spec3 antiserum would then be abolished because of the specificity of the antiserum to the first 14 amino terminal residues of the protein

Gastrula stage embryos were treated with trypsin at a final concentration of 0 25 mg/ml for 1 or 5 minutes before fixation and immunofluorescence staining Trypsin treatment did not decrease the intensity of staining on cilia but resulted in the disappearance of the characteristic beaded pattern and the appearance of kinks along the cilia (compare Fig 9a and b)

Trypsin treatment did not disrupt membranes as shown by the fact that treated live embryos excluded Trypan blue Moreover, embryos continued to swim even after 30 minutes in trypsin, showing that the embryos remained viable and that their cilia retained their motor competence

Since trypsin treatment did not reduce the intensity of staining on cilia, the amino terminal region of Spec3 is probably not accessible for cleavage However, the disruption of the beaded pattern suggests that trypsin modifies the spatial organization of the membrane such that there is a change in the accessibility of the Spec3 antigen to the antiserum Alternatively, Spec3 could be a component of a membrane-axoneme structure to which a modification of

the membrane would be transmitted, affecting its affinity for the Spec3 antiserum.

IMMUNOCYTOCHEMICAL STAINING OF CILIA AND AXONEMES

When whole embryos were fixed and labeled by immunofluorescence, cilia were stained with a beaded pattern that persisted in cilia which fell off embryos during processing (Fig 10a). Cilia could also be fixed and stained after being removed from live embryos by hypertonic shock but the beaded pattern was no longer present and the labeling was less intense than on whole embryos (Fig 10b) When the membrane of such cilia was removed by detergent extraction before immunocytochemistry, the pattern of staining was not altered, suggesting that Spec3 is localized on the axoneme (Fig 10c)

Removal of cilia from embryonic cells seems to cause a reorganization of Spec3 in cilia as seen by the disappearance of the beaded pattern A possibility is that part of the protein resides in the soluble matrix between the plasma membrane and the axoneme Upon deciliation, it is likely that the membrane remains unsealed for some time, letting the soluble proteins leak out This explanation could account for the reduction in the intensity of staining in isolated cilia compared to cilia fixed when still attached to embryos However, immunofluorescence staining of demembranated cilia and immunoblotting of proteins from ciliary fractions (see next section) suggest that most of Spec3 is associated with the axoneme

IMMUNOBLOTTING WITH THE SPEC3 ANTISERUM

The predicted molecular weight of the Spec3 protein is about 21.6 kilodalton, as deduced from the open reading frame of the cDNA sequence (Eldon et al, 1987). When ciliary proteins from S purpuratus were immunoblotted and incubated with the Spec3 antiserum followed by radioiodinated protein A, an intensely labeled band was observed Sometimes, this band was resolved into a pair of bands (Fig. 11a). The apparent molecular weight of the stained protein is much larger than that predicted for Spec3. On 4-12% polyacrylamide gradient gels (Fig. 18a, lane 6), the protein migrated more slowly than dyneins which are reported to have a molecular weight of 350 kD or more (Bell et al 1982 and Fig.18a, lane 1). Thus, Spec3 seems to form large aggregates that cannot be dissociated by reductive extraction in the presence of boiling or cold SDS Reduction and alkylation of proteins with N-ethyl-maleimide before electrophoresis did not change the mobility of these aggregates (Fig. 11b), indicating that cysteine cross-links are not responsible for their persistence The antiserum cross-reacted with egg albumin which was used as a molecular weight marker (Fig. 18a, arrowhead) Interestingly, an anti-albumin antiserum labels the Golgi of liver cells (Geuze et al, 1981)

Immunoblotting revealed an enrichment of the Spec3 antigen in ciliary extracts compared to whole embryos (Fig 17c, compare lanes 3 and 4) It is difficult to calculate the relative amounts of Spec3 in cilia and Golgi complexes because ciliary proteins account for only about 1/500 of total embryonic proteins. Therefore, a direct quantitative comparison between ciliary proteins and proteins from

deciliated embryos would require loading 500 times more proteins from the latter sample, overloading the gel.

The developmental profile of Spec3 analyzed on immunoblots confirmed the results obtained by immunostaining of whole embryos. Spec3 became more abundant on cilia starting at the gastrula stage (Fig. 11 b, c). However, the protein was detected earlier on blots than by immunocytochemical staining of whole embryos. Fig. 11c, lane 1 shows that Spec3 was detected in pre-hatching cilia of blastulae on blots whereas at that stage, no staining was detected on cilia by immunofluorescence (see Fig. 5).

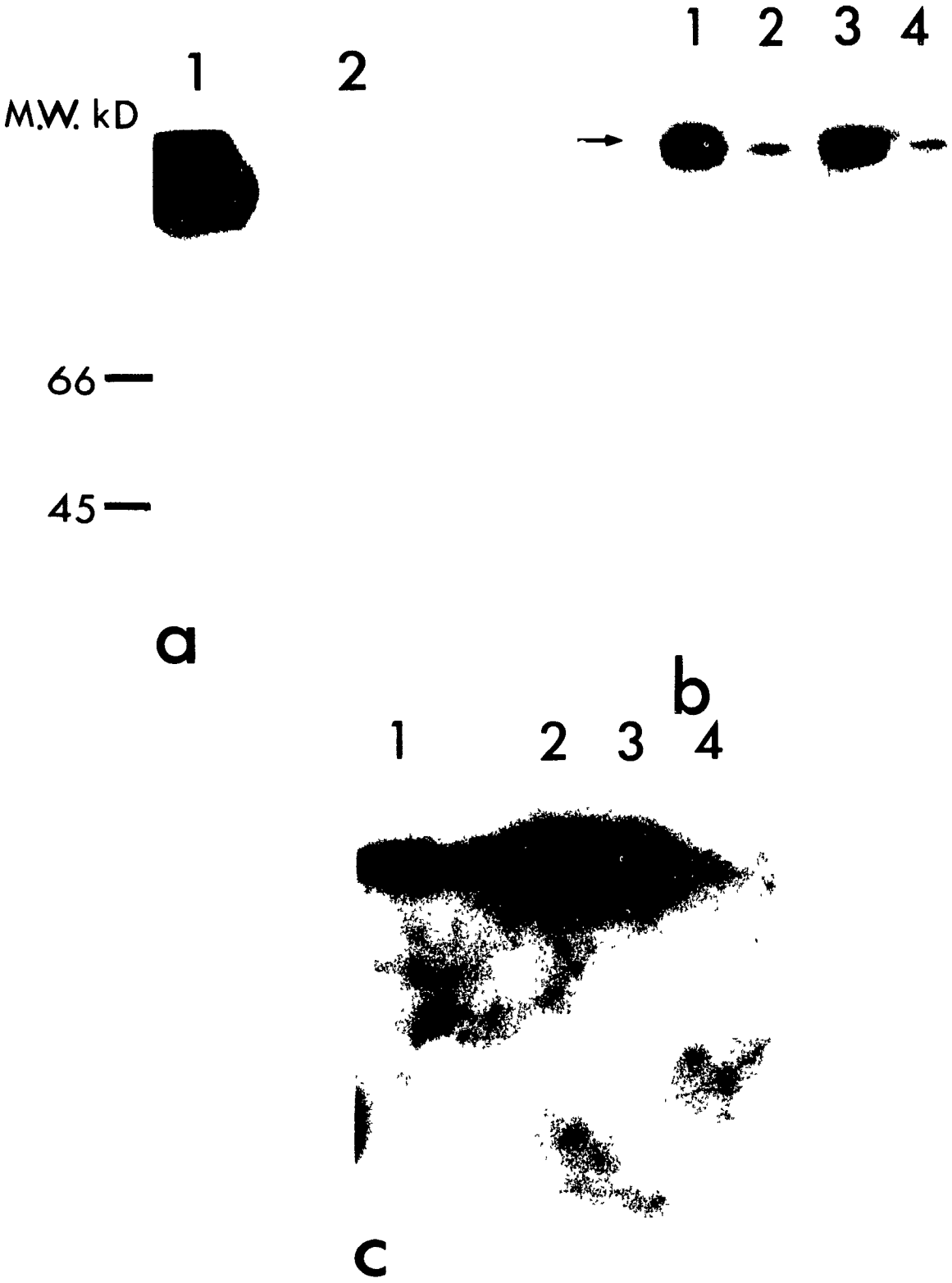
Since cilia and flagella share many proteins, the presence of Spec3 on flagella was investigated. Immunoblotting of sperm flagellar proteins with the Spec3 antiserum did not reveal any labeled band (Fig. 11c, lane 4) and sperm flagella did not stain by immunofluorescence with the Spec3 antiserum (data not shown), suggesting that Spec3 is not a general axonemal protein but is specific to ectodermal cilia

Figure 11. **Immunoblot analysis using the Spec3 antiserum.**

a) The Spec3 antiserum labels a band of very high apparent molecular weight. Immunoblots of duplicate 10% acrylamide mini-gels of prism ciliary proteins treated with the Spec3 antiserum (lane 1) and the preimmune serum (lane 2) .

b) Spec3 accumulates in cilia after ciliogenesis is completed. Reduction and alkylation do not change the mobility of Spec3 on gels. Equal amount of proteins were loaded on each lane. Lanes 2 and 4. hatching blastula stage ciliary proteins. Lanes 1 and 3. prism stage ciliary proteins. Lanes 3 and 4: proteins were reduced and alkylated before electrophoresis. The arrow points to the top of the gel

c) Developmental profile of the Spec3 protein on cilia and flagella. Lane 1 contains twice as much protein as lanes 2, 3 and 4. Lane 1. blastula cilia before hatching Lane 2. gastrula cilia Lane 3 pluteus cilia Lane 4: sperm flagellar proteins.


1 2 3 4
1 2
M.W. kD
66
45
a
b
1 2 3 4
c

Monensin treatment

In mammalian cells, the ionophore monensin prevents secretion of proteins from the Golgi apparatus, resulting in swelling of the complex (Tartakoff, 1983). When prism embryos were treated with monensin at concentrations ranging from 10^{-5} to 10^{-7} M for 20 minutes, the Golgi complexes became smoother and larger in shape and appeared more brightly stained When embryos were treated for a longer period of time, staining of the cilia declined gradually and became undetectable by 4 hours (Fig. 12). Even after 5.5 hours in monensin, embryos were able to move. These results suggest that Spec3 is secreted from the Golgi to the cilia and imply that Spec3 turns over rapidly on cilia. They also indicate that beating of cilia does not depend on the presence of the Spec3 protein.

Deciliation and monensin treatment

Since the Spec3 protein appears to transit through the Golgi to the cilia, it was interesting to determine if monensin treatment prevents new growth of cilia after deciliation.

Deciliation

Prisms were deciliated by hypertonic shock and the cilia were allowed to regenerate. Although no movement was detectable in embryos until 60 minutes after deciliation, growth of cilia was detected after 30 minutes when embryos were stained with the Spec3 antiserum At that time, most of the stain concentrated at the tip of the

growing cilium appearing as a dot on top of the Golgi (Fig. 13a). At 45 minutes, the growing cilia stained over their entire length (Fig 13b) but the beaded pattern of staining appeared only at about 60 minutes (Fig. 13c,d), when embryos started to oscillate due to ciliary movement. After 150 minutes, these embryos moved like the controls although their cilia did not grow to their full size until after 300 minutes of regeneration.

Deciliation followed by monensin treatment

When embryos were deciliated and allowed to regenerate their cilia in the presence of 10^{-5} M monensin for 90 minutes, the Golgi complexes of aboral ectoderm cells swelled and ciliary growth was detectable by staining with the Spec3 antiserum (Fig. 14b). However, cilia had the length and appearance of 30 minutes controls (compare Fig. 14b and 13a).

The staining of growing cilia in the presence of monensin could be due to the presence of the antigen downstream from the Golgi at the time of monensin addition.

Figure 12 **Effect of monensin on the distribution of Spec3.**

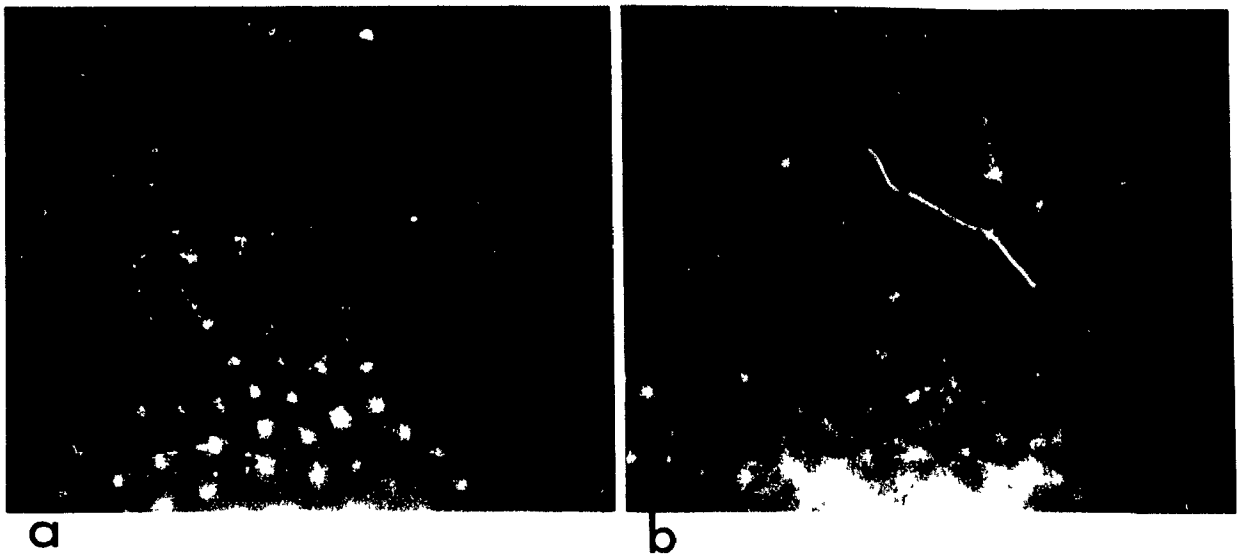
Analysis by immunofluorescence staining.

a) Control prism embryo.

b) Prism treated with monensin for 1 5 hour. Staining of the cilia is fainter.

c) Prism treated with monensin for 3 hours.

d) Prism treated with monensin for 5.5 hours


a
b

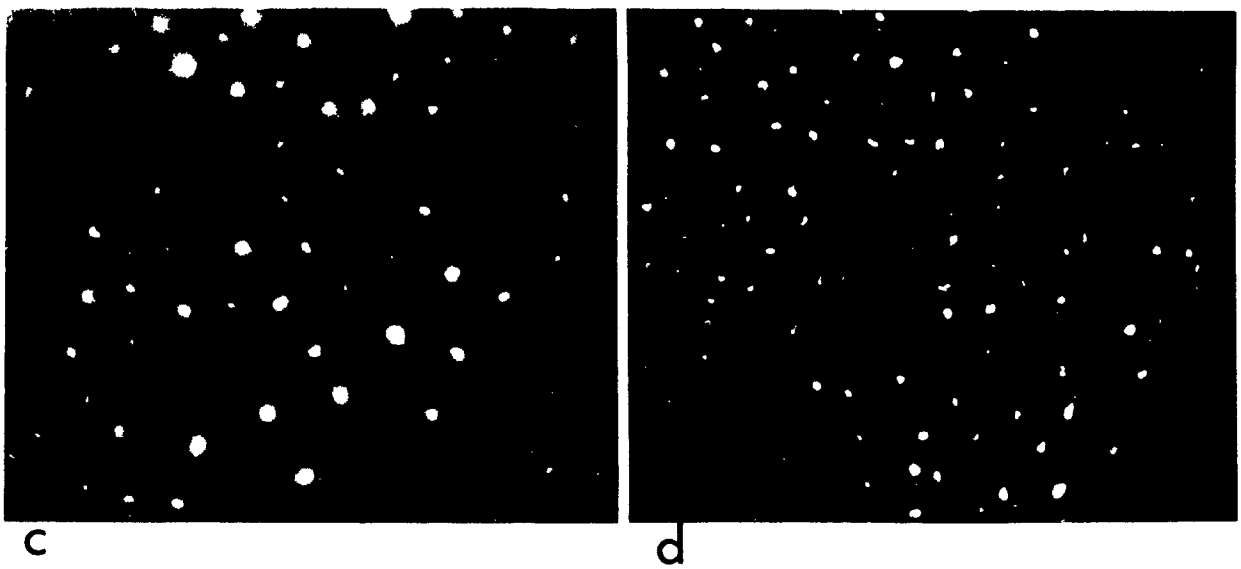

c
d

Figure 13. **Distribution of Spec3 in prism stage embryos during cilia regeneration**

Analysis by immunofluorescence staining (x580)

a) Immunofluorescence staining of an embryo 30 minutes after deciliation Arrow points to growing cilium.

b) Pattern of staining 45 minutes after deciliation.

c) Pattern of staining 90 minutes after deciliation.

d) Pattern of staining 105 minutes after deciliation

a
b
c
d

Figure 14 **Effect of deciliation and monensin treatment on the distribution of Spec3.**

Analysis by immunofluorescence staining.

a) Control embryo without deciliation or monensin treatment (x580)

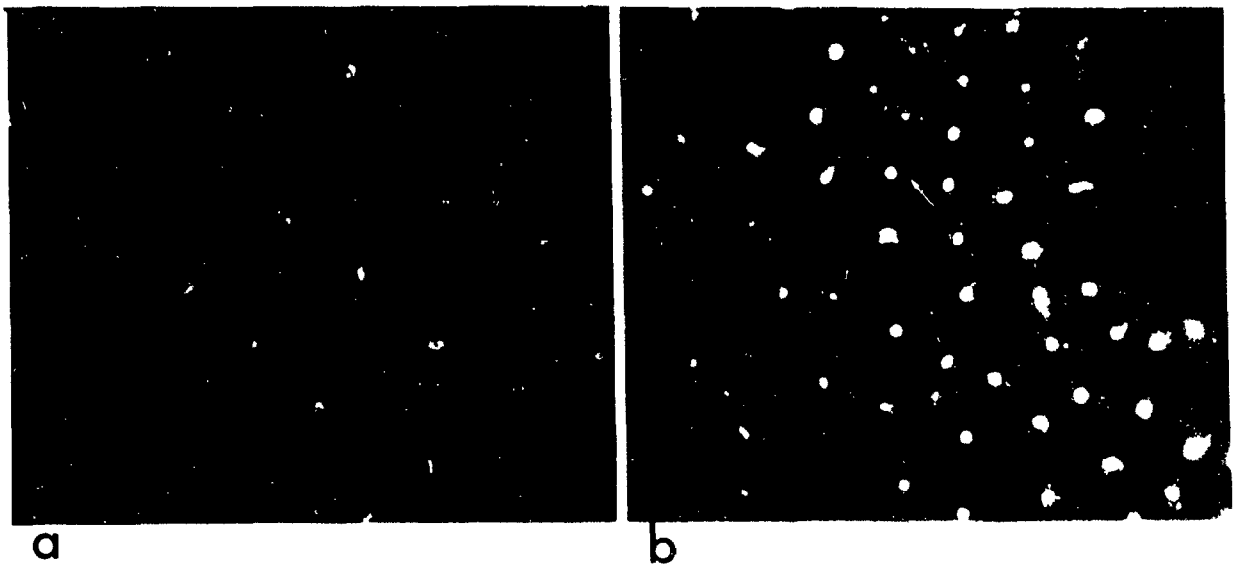
b) Embryo deciliated and treated with monensin for 90 minutes during ciliary regeneration (x370). Arrow points to growing cilium

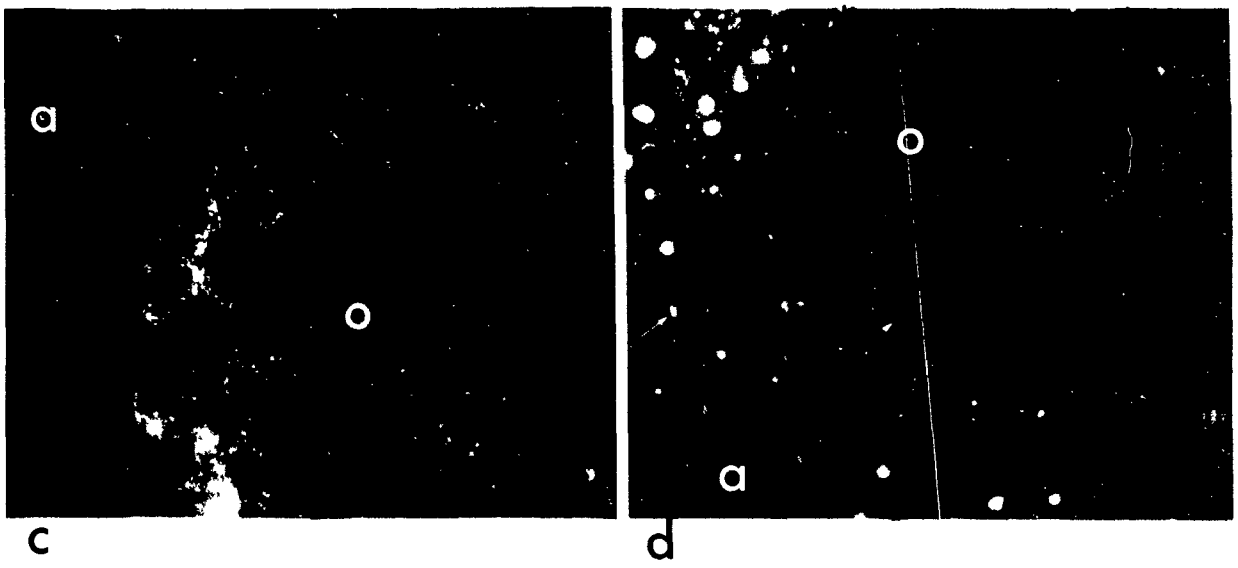
c) Control oral ectoderm of a prism stage embryo (x580)

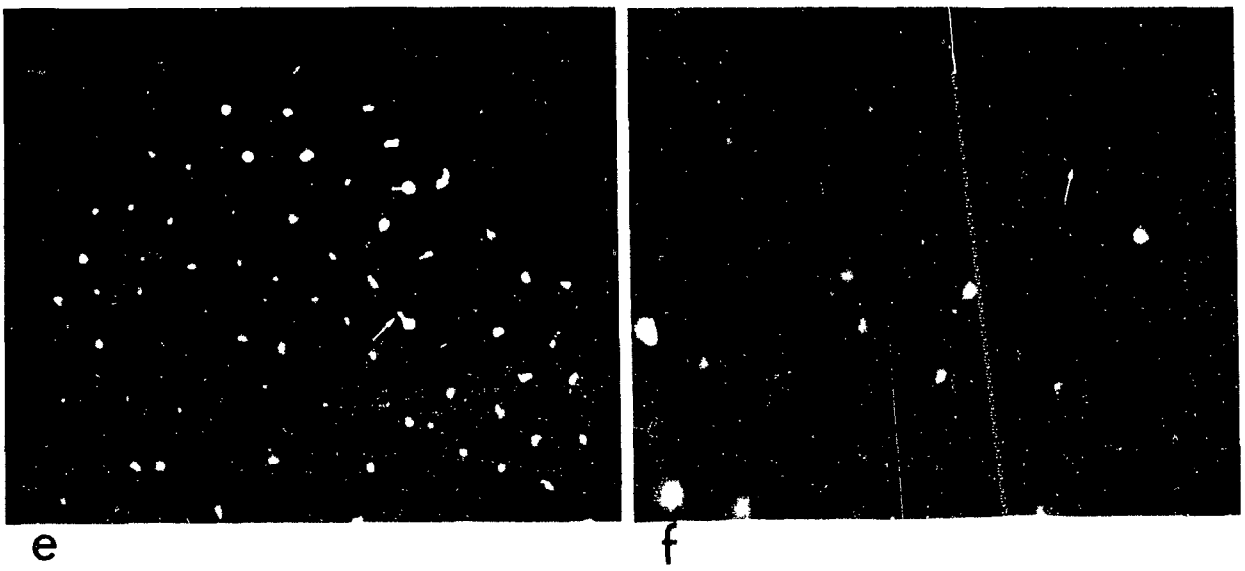
d) Oral ectoderm of an embryo treated as in (b) There is no bright structure at the base of growing oral cilia Compare arrowhead with arrow O oral ectoderm, a aboral ectoderm Photography of panels (c) and (d) was done under the same exposure conditions (x580)

e) Embryo treated with monensin for 90 minutes prior to deciliation and regeneration in the presence of monensin Regeneration was allowed for 3 hours. Arrow points to a growing cilium (x370)

f) Embryos treated as in (e) Regeneration was allowed for 4 hours (x850) Arrow points to a growing cilium


a
b


a
o
o
a
c
d

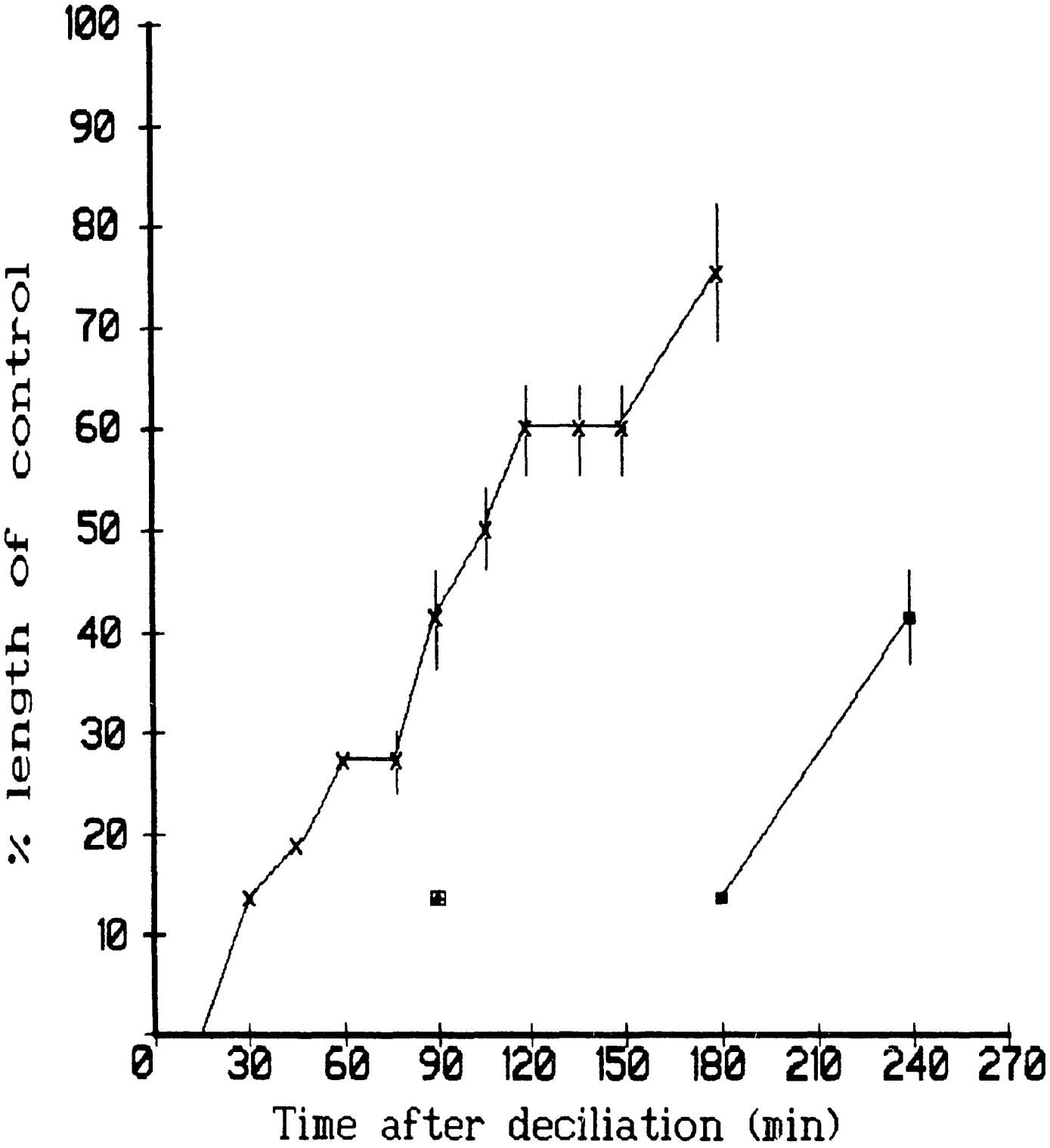

e
f

To test that possibility, embryos were treated with 10^{-5} M monensin for 90 minutes before deciliation and regeneration in the presence of monensin. There was no detectable growth of cilia until 3 hours after deciliation (Fig. 14e) and after 4 hours, cilia had the same length as 90 minutes controls (compare Fig 14f and 13c) However, those embryos were not moving.

These results suggest that secretory activity of the Golgi apparatus is essential for normal ciliary growth. The observed limited growth could be due to a leaky effect of the drug Interestingly, embryos treated with monensin before deciliation did not move even if their cilia were growing Monensin toxicity is not sufficient to explain this result since monensin treated embryos that had not been deciliated were moving after the same treatment However, it could be that a protein needs to be processed via the Golgi in order to allow growing cilia to move and that under the experimental conditions, that protein could not be exported to cilia in sufficient quantity

Figure 15. **Time course of ciliary growth.**

Ciliary growth was measured after deciliation, deciliation followed by treatment with 10^{-5} M monensin, treatment with 10^{-5} M monensin for 1.5 hour prior to deciliation and regeneration in the presence of 10^{-5} M monensin



X Deciliation

■ Deciliation followed by monensin treatment

■ Monensin treatment before deciliation. Regeneration in the presence of monensin.

Fig 15 illustrates the growth of cilia under the various conditions mentioned above

Experiments with monensin also confirm that there is no staining of Golgi in the cells of the oral ectoderm When prisms were deciliated and incubated with monensin, cilia of the oral ectoderm started to grow but no swollen Golgi were observed at their proximal ends whereas such swelling was obvious in the aboral area (Fig 14c,d)

Effects of drugs on the distribution of Spec3

Puromycin

Since the Spec3 protein appears to turn over rapidly on cilia, it was interesting to look at the effect of protein synthesis inhibitors on the prevalence of Spec3 on cilia Blastulae were treated with puromycin at a concentration of 100μM for 4 hours This concentration inhibits reversibly 95% of protein synthesis as measured by ^{35}S-methionine incorporation (data not shown) During the treatment, fluorescence disappeared gradually from the Golgi and became concentrated in a globule at the tip of each cilium (Fig 16b) When embryos were removed from puromycin for 2 hours, cilia regained their normal shape but staining of the Golgi did not reappear (Fig 16c)

Experiments described above using monensin suggest that Spec 3 turns over rapidly on cilia Therefore, I expected that after protein synthesis inhibition, the intensity of staining of Golgi and cilia with the Spec3 antiserum would decrease gradually and that by

allowing pulses of protein synthesis, I could obtain an estimate of the turnover rate of Spec3 However, results from protein synthesis inhibition differ from results from monensin treatment in that they suggest that turnover takes place in the Golgi rather than on cilia When embryos were treated with puromycin, Golgi staining disappeared and cilia staining increased as if proteins in the Golgi were quickly transported to the tip of the cilia One possible interpretation is that the distribution of Spec3 and possibly other proteins on cilia results from a balance between anterograde and retrograde transport and that a retrograde component of that transportation system is a labile protein When protein synthesis was restored, Spec3 rapidly recovered its normal arrangement in the cilium but not in the Golgi This observation is consistent with the reestablishment of a retrograde transport system. Protein synthesis recovery was probably not long enough to allow accumulation of Spec3 in the Golgi

Figure 16 **Effect of tunicamycin, puromycin and a proline analogue on the distribution of Spec3.**

Immunocytochemical analysis (x370)

a) Control mesenchyme blastula (4 hours after hatching)

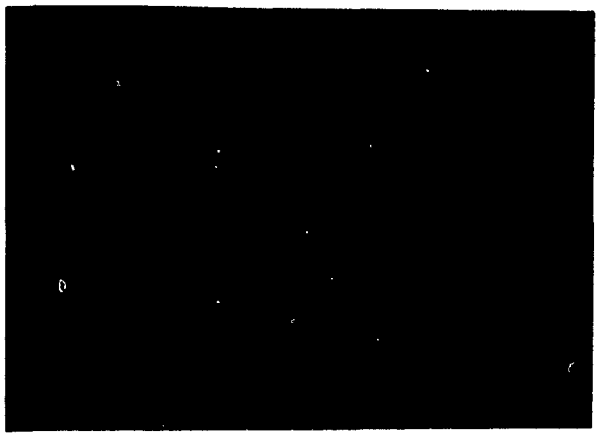
b) Hatching blastula treated with 100 μg/ml puromycin for 4 hours

c) Embryo treated as in (b) but cultured for 2 extra hours in the absence of puromycin

d) Hatching blastula treated with 10 μg/ml tunicamycin for 4 hours (until the mesenchyme blastula stage)

e) Embryo treated with 500 μg/ml L-azetidine-2-carboxylic acid for 14 hours

a



b

c

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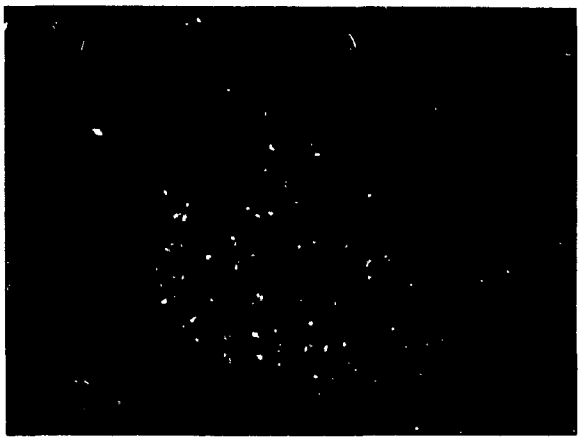


e

Treatment of embryos with a proline analogue

The amino acid composition of the deducted sequence of the Spec3 protein is unusual. It contains 28 proline residues, all located in the first 138 amino terminal residues, 2 being included in the N-terminal peptide used to generate the antiserum (Eldon et al, 1987). Since proline causes polypeptide chains to bend, the abundance of proline residues is probably determinant for the tertiary structure of Spec3, its antigenicity and its function in the cell

L-azetidine-2-carboxylic acid, a proline analogue, can be incorporated *in vivo* to high levels into newly synthesized plant proteins (Fowden and Richmond, 1963). Because of its structure, this analogue of proline should turn an α-helix through an angle about 15° smaller than proline (Fowden and Richmond, 1963), thereby altering the tertiary structure of the protein Proline replacement by L-azetidine-2-carboxylic acid might prevent proper localization of Spec3, impede its normal function in the cell, or destroy its affinity for the antiserum.

When blastulae that were just starting to move were treated with L-azetidine-2-carboxylic acid for 14 hours, there was no effect on morphology or movement at concentrations ranging from 10 to 100 μg/ml At a concentration of 500μg/ml, most embryos were not moving normally but were oscillating rather than swimming However, when labeled by immunofluorescence, such embryos displayed the normal pattern of staining for the Spec3 protein (Fig 16e), suggesting that proline replacement did not alter the localization of the protein and its affinity for the antiserum

One possibility is that proline replacement caused a conformational change in Spec3 that affected its function without interfering with its localization and its reactivity with the antiserum The abnormal movement of L-azetidine-2-carboxylic acid treated embryos is consistent with a role of Spec3 in normal movement However, since the incorporation of L-azetidine-2-carboxylic acid into proteins was not estimated, it is possible that this proline analogue is not incorporated in proteins of sea urchin Abnormal movement of treated embryos could be due to a general toxic effect of the drug

Tunicamycin

The cDNA sequence of Spec3 reveals a potential N-linked glycosylation site in the protein (Eldon et al, 1987) Spec3 also appears to transit in the Golgi before being localized in the cilia (see previous section) Thus, Spec3 might be glycosylated during its passage through the Golgi apparatus, consistent with its apparent large size on SDS gels (see section on immunoblotting) The drug tunicamycin blocks the first step in the synthesis of sugars involved in glycosylation (Elbein, 1987) If glycosylation is required for the localization of Spec3 on cilia, tunicamycin treatment could lead to a decrease in staining of cilia with time

Blastulae were treated with tunicamycin at concentrations of 0 1, 1 and 10 μg/ml for 4 hours No effect could be detected at concentrations less than 10μg/ml However, after 4 hours at that concentration, very few cilia or Golgi could be stained with the Spec3 antiserum (Fig 16d) suggesting that glycosylation of Spec3 or

other proteins is essential for the proper localization of the Spec3 protein However, in many cases, proteins that have not been glycosylated normally are still properly localized Alternatively, since some glycoproteins are degraded more rapidly in cells treated with tunicamycin (Elbein, 1987), rapid decay of Spec3 might explain its decline on Golgi and cilia

SPECIFICITY OF THE SPEC3 ANTISERUM

Several experiments from W H Klein's laboratory verify the specificity of the Spec3 antiserum for the Spec3 protein I have repeated some of them and confirmed their results

By staining embryos with decreasing concentrations of the primary antiserum, it should be possible to determine the lowest concentration at which staining is still detectable Then, preincubation of the primary antiserum with the Spec3 N-terminal peptide against which it was initially raised should prevent staining of embryos because of competition of the Spec3 protein and the peptide for binding to antibodies

Figure 17 **Specificity of the Spec3 antibody.**

a) Immunofluorescence staining of embryos with diluted primary antiserum Arrow points to a stained cilium. (x370)

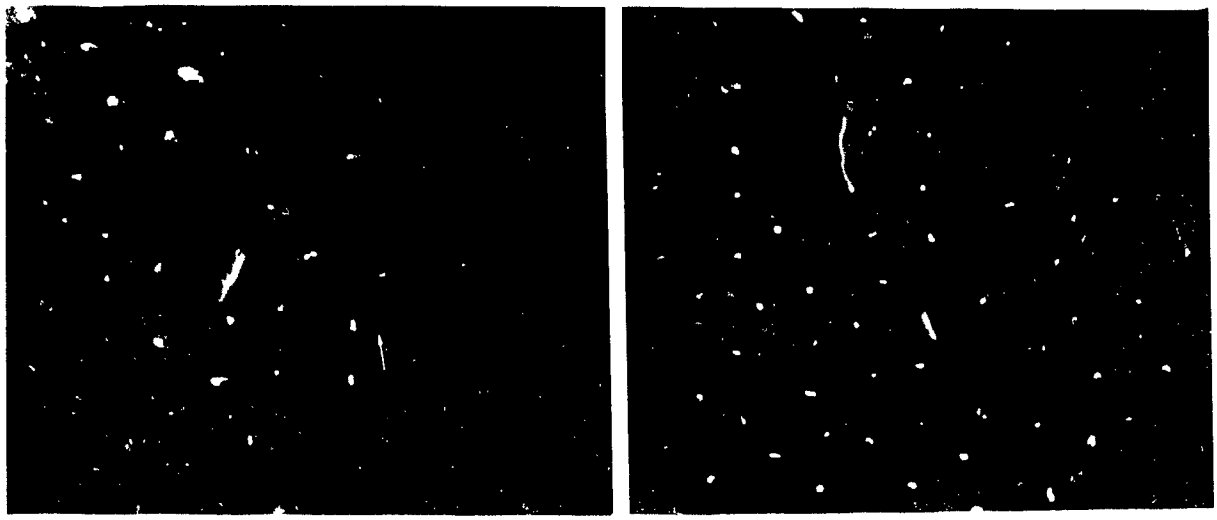
b) Embryos treated as in (a) but primary antiserum was preincubated with the N-terminal peptide used to generate it Cilia are no longer stained but Golgi is. (x370)

c) Immunoblot of protein extracts separated by electrophoresis on a 10% acrylamide gel and incubated with the Spec3 antiserum followed by radioiodinated protein A All lanes contain 10μg of proteins except lane 6 which contains 2 5μg Lane 1 deciliated embryos allowed to regenerate their cilia for 2 5 hours. Lane 2 embryos just deciliated Lane 3 cilia Lane 4 whole embryos Lane 5 egg proteins Lane 6 deciliated embryos allowed to regenerate their cilia for 30 minutes

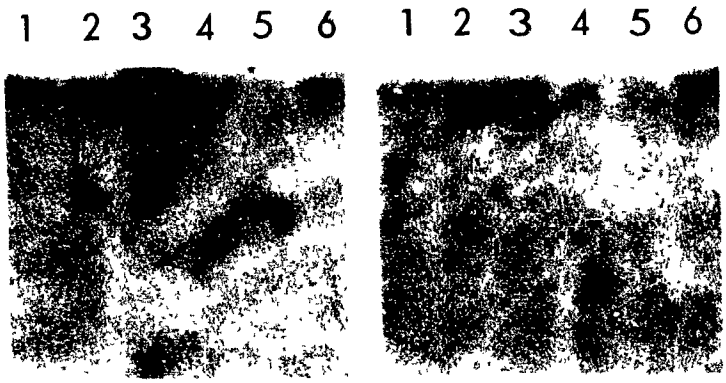
d) Replica of (c) The Spec3 antiserum was preincubated with the N-terminal peptide used to generate it

e) Immunoblot of protein extracts incubated with the Spec3 antiserum followed by iodinated protein A Lane 1 40μg of proteins from deciliated embryos allowed to regenerate their cilia for 2 5 hours Lane 2 5μg of ciliary proteins

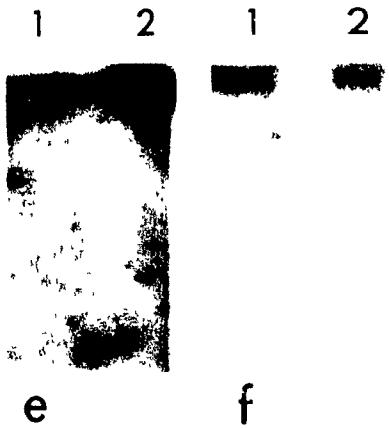
f) Replica of (e) treated as in (d)



a b



c d



e f

When the concentration of primary antiserum was lowered to 10μg/ml, cilia barely stained but the Golgi was still very bright (Fig 17a) Preincubation of the antiserum with the N-terminal peptide resulted in the disappearance of fluorescence on cilia but not on the Golgi (Fig 17b)

Immunoblotting gave similar results When the antiserum was preincubated with the N-terminal peptide, detection of Spec3 protein from ciliary extracts with radio-iodinated Protein A was practically abolished (compare Fig 17c and d, lane 3, 17e and f, lane 2) For blots of proteins from whole embryos (Fig 17 c, d, lane 4), deciliated embryos (lane 2), and deciliated embryos allowed to regenerate their cilia for 30 minutes (lane 6) or 2 5 hours (lane 1), preincubation of the antiserum with the peptide caused a decrease in the intensity of the signal compared to treatment with the intact serum (compare Fig 17 c and d)

Fig 17 e, f illustrates a similar experiment In lane 1 (deciliated embryos allowed to regenerate their cilia for 2 5 hours), I loaded 8 times the amount of proteins as in lane 2 (ciliary proteins) When the Spec3 antiserum was pre-incubated with the N-terminal peptide, most of the label in lane 2 disappeared (Fig 17f) In lane 1, a larger proportion of the antigen did not compete with the peptide This competition resistant fraction possibly corresponds to the population of Spec3 molecules that are localized in the Golgi complex and did not compete with the peptide in immunofluorescence staining (Fig 17b)

These results suggest that there are two types of molecules that can be detected with the Spec3 antiserum One of them is located

on cilia and competes with the peptide. The other is a Golgi protein that has the same electrophoretic mobility as the ciliary one but does not compete with the peptide. The two types of proteins share epitopes that react with the antiserum. It is difficult to attribute their differences to structural features that could be resolved by SDS gel electrophoresis because they both migrate as if their molecular mass was very large It is not easy to detect small differences in size between such large aggregates Another electrophoresis system might be able to distinguish between the two types of molecules

FRACTIONATION OF CILIA

Fig 20 illustrates the different steps in the fractionation scheme.

That Spec3 seems to transit in the Golgi before being localized in cilia suggests that it might be a membrane protein It should then fractionate with ciliary membranes rather than with axonemes

Figure 18 **Fractionation of ciliary proteins.**

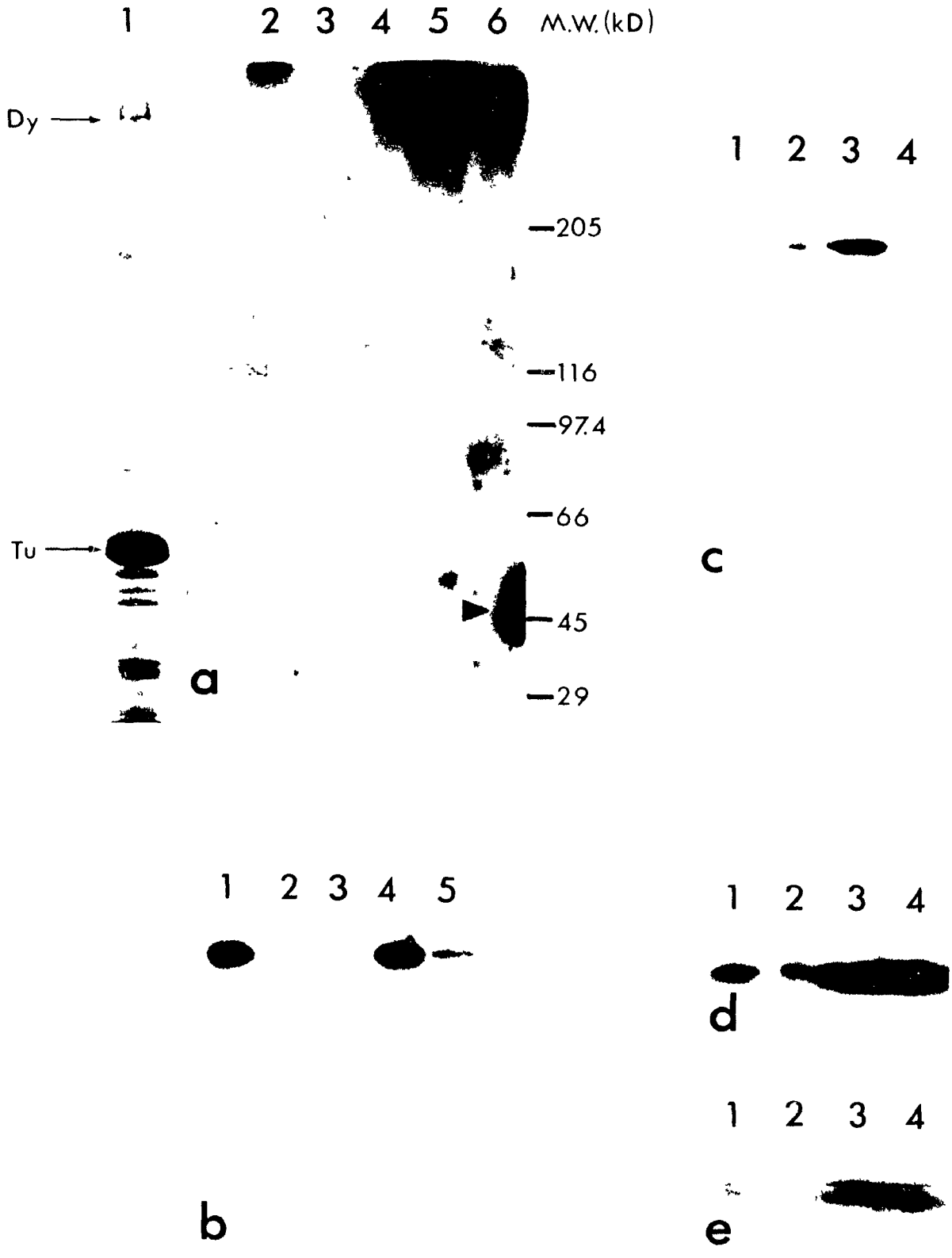
Analysis by immunoblotting

a) The Spec3 antigen does not fractionate with membranes. Immunoblot of a 4-12% polyacrylamide gradient gel of fractions of ciliary proteins The blot was treated with the Spec3 antiserum followed by radioiodinated protein A Stoichiometric amounts of cilia were loaded on each lane except lane 6 Lanes 2 and 3· Triton X-114 fractionation of membrane proteins Lane 2 integral membrane proteins (detergent soluble fraction) Lane 3 non-integral membrane proteins (aqueous fraction) Lane 4 Total membrane proteins solubilized with Triton X-100 Lane 5 Axonemal proteins Lane 6 proteins from whole cilia Arrow head shows cross hybridization with egg albumin, a molecular weight marker Lane 1 replica of lane 6 stained with Coomassie blue Arrows point to dynein and tubulin

b) Spec 3 is not soluble in a high salt buffer Ciliary proteins were fractionated by the method of Dentler (1988) into integral membrane proteins (detergent soluble, lane 3) and non integral membrane proteins (aqueous fraction, lane 2) The insoluble axonemal proteins were then subjected to a high salt extraction according to Bell et al (1982) The high salt soluble fraction was loaded on lane 5 and the insoluble axonemal proteins were loaded on lane 4 Lane 1 contains total ciliary proteins Stoichiometric amounts of cilia were loaded on each lane

c) Spec3 is insoluble in 0 5% Sarkosyl Ciliary proteins were separated into membrane proteins(lane 1) and high salt soluble

proteins (lane 2) according to Stephens (1986) The insoluble axonemal proteins were separated into Sarkosyl insoluble (lane 3) and soluble (lane 4) proteins according to Stephens, 1978 Stoichiometric amounts of cilia were loaded on each lane d) and e) Spec3 is partly solubilized by a Sarkosyl-urea extraction Ciliary protein were treated as in (c) but urea was added to the Sarkosyl extraction solution (Linck et al, 1985) Lane 1: membrane proteins Lane 2 high-salt soluble fraction Lane 3: Sarkosyl-urea soluble fraction Lane 4 Sarkosyl-urea insoluble fraction Stoichiometric amounts of cilia were loaded on each lane Panel (d) and (e) are two different exposures of the same blot


1 2 3 4 5 6 M.W.(kD)
Dy
205
116
97.4
66
Tu
45
29
a
1 2 3 4
c
1 2 3 4 5
b
1 2 3 4
d
1 2 3 4
e

Ciliary membrane proteins were prepared according to two methods The method of Stephens (1986) separates membrane proteins from axonemes by extraction in the presence of the detergent Triton X-100 The method of Dentler (1988) fractionates ciliary proteins into three subfractions integral membrane proteins, other membrane proteins, and axonemal proteins, based on differential solubilization in the detergent Triton X-114 Both methods gave the same result Spec3 did not fractionate with the membrane proteins but stayed in the axonemal fraction (Fig 18a, lanes 2,3,4,5) although in some experiments, a small fraction of Spec3 separated with the detergent phase (Fig 18a, lanes 2, 4, 18 d, lane 1) These results are consistent with immunocytochemical data (see above) where cilia stripped of their membranes stained with the same pattern as hole cilia The fact that in some experiments, part of Spec3 separated with the detergent phase could be explained by the localization of a subset of Spec3 molecules in the soluble matrix between the membrane and the axoneme This possibility was also invoked to explain the pattern of staining of isolated cilia (see section on immunocytochemical staining of isolated cilia) This soluble component should co-fractionate with the membranes

Dynein arms are axonemal structures involved in movement They contain polypeptide subunits with very high molecular weights, ranging from 300 to 500kD (Bell et al, 1982) Since Spec3 appears coincidentally with changes in ciliary movement and it migrates like a very large polypeptide on SDS gels, I was wondering whether it was itself a dynein or was associated with the dynein arm The axonemal fraction was thus extracted with a high-salt buffer according to Bell

et al (1982) or Stephens (1986) These procedures should solubilize half of the dyneins of ciliary axonemes (Stephens hypothesizes that it might solubilize the outer dynein arm), one member of the central pair of microtubules and all of the B-subfiber of the outer doublet microtubules (Stephens, 1986)

In both instances, Spec3 stayed with the insoluble fraction (Fig 18b, lane 4, 18c, lane 3) which should contain among other proteins, tektins and tubulins If inner and outer dynein arms solubilize randomly, these results suggest that Spec3 is not a dynein or a protein associated with dyneins Alternatively, if the high salt soluble half of dyneins is indeed the outer arm, Spec3 could still be associated with the inner arm

The protofilaments that join the A and B subfibers of the outer doublet microtubules are resistant to many extraction methods (Stephens, 1978, Linck et al 1985) Their major component is the protein tektin

Stephens (1978) used 0 5% Sarkosyl to solubilize all the proteins from the A-tubule of cilia except those from the junctional protofilaments When this procedure was used on the high salt insoluble pellet (see above), most of the tubulins were solubilized (Fig 19a, lanes 4,7), but not Spec3 (Fig 18c)

Extraction in 0 5% Sarkosyl and 2 5M urea (Linck et al, 1985), solubilized even more proteins, including most of the tubulins (Fig 19b, lane 4) and immunoblots revealed that about half of Spec3 was solubilized and half remained in the insoluble fraction (Fig 18d, e) It was the first procedure that led to at least partial solubilization of Spec3

Together, these results show that Spec3 is very resistant to many extraction systems and most of it seems to be strongly associated with the core of the axoneme. Clearly, it does not fractionate with membranes The results suggest that it is not associated with the dynein arms although only analysis at the electron microscopic level might resolve that point Since Spec3 is partially solubilized by treatment with Sarkosyl and urea, it does not seem to be tightly associated with tektins One possibility is that it is a microtubule associated protein that is associated with the subset of microtubule that is the most resistant to extraction


a
1 2 3 4 5 6 7 8 9
M.W (kD)
205
116
97.4
66
45
29
Tu
b
1 2 3 4 5 6 7 8
MW (kD)
66
45
36
29
24
20.1
Tu

Figure 19. **Electrophoretic analysis of the ciliary fractions.**
a) Silver stained 4-12% acrylamide gradient gel of fractionated ciliary proteins. Stoichiometric amounts of cilia were loaded except in lane 1 which contains 10 times less cilia. Lane 1: whole cilia. Lane 2: Triton X-100 extracted membrane proteins Lanes 3, 4 and 5 result from the same initial sample. Lane 3· high salt soluble proteins extracted according to Bell et al (1982). Lane 4· sarkosyl soluble proteins from the pellet left after solubilization of the proteins on lane 3. Lane 5: sarkosyl insoluble pellet. Lanes 6, 7 and 8 result from the same initial sample. Lane 6. high salt soluble proteins extracted according to Stephens (1986). Lane 7 sarkosyl soluble proteins from the pellet left after solubilization of the proteins on lane 6 Lane 8· sarkosyl insoluble pellet Lane 9: molecular weight markers. arrows point to tubulins
b) Silver stained 10% acrylamide mini-gel of fractionated ciliary proteins Stoichiometric amounts of cilia were loaded on each lane (except lane 1 which contains ten times less). Lane 1: whole cilia. Lane 2. Triton X-100 solubilized membrane proteins Lane 3 high salt soluble proteins (Stephens, 1986) Lane 4. Sarkosyl urea soluble proteins. Lane 5 Sarkosyl urea insoluble proteins. Lanes 6 and 7 are the same as lanes 4 and 5 from (a) Arrows point to tubulins.

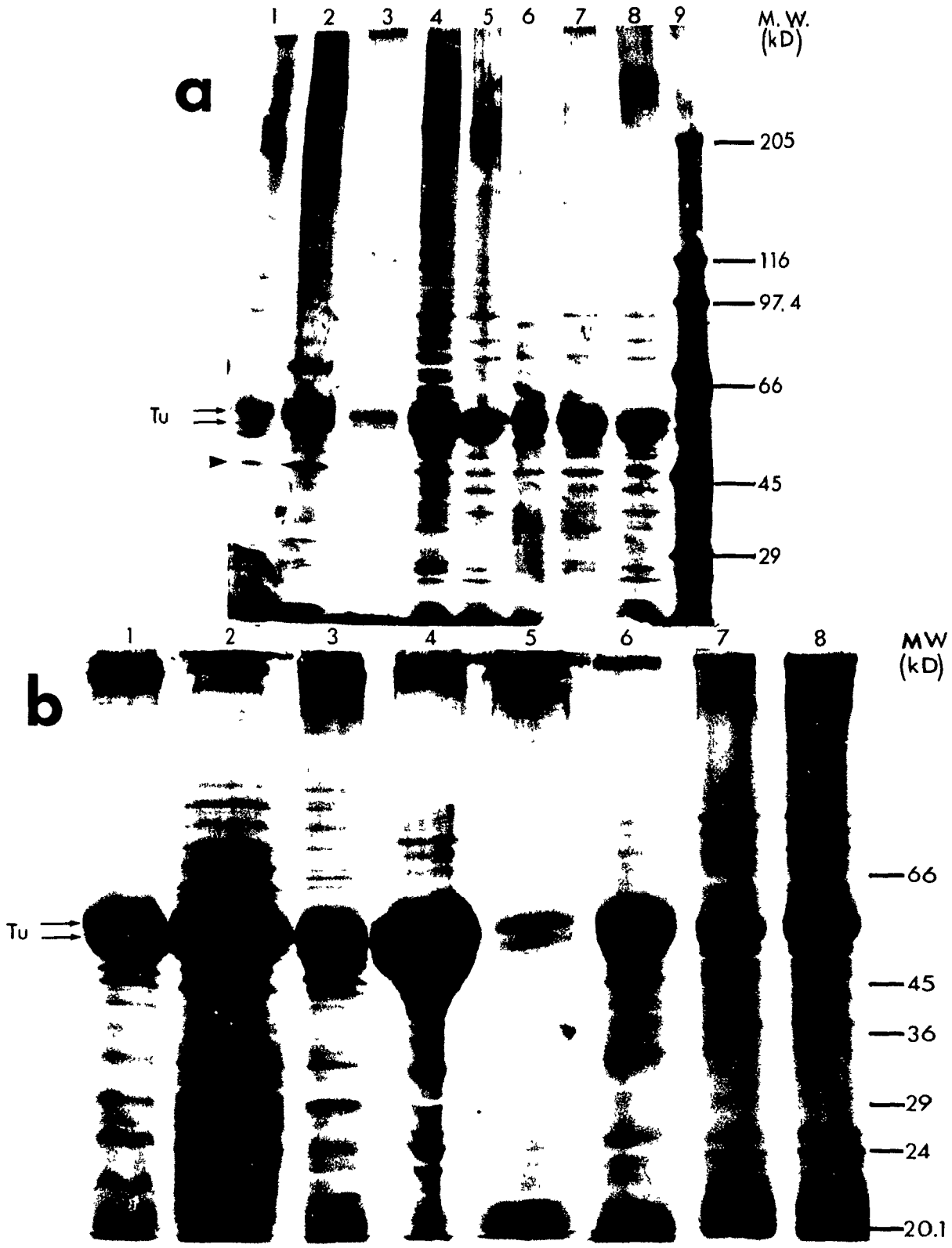

a
1 2 3 4 5 6 7 8 9
M. W. (kD)
205
116
97.4
66
45
29
Tu
b
1 2 3 4 5 6 7 8
MW (kD)
66
45
36
29
24
20.1
Tu

Figure 20. Steps in the fractionation of cilia.

Fractionation scheme

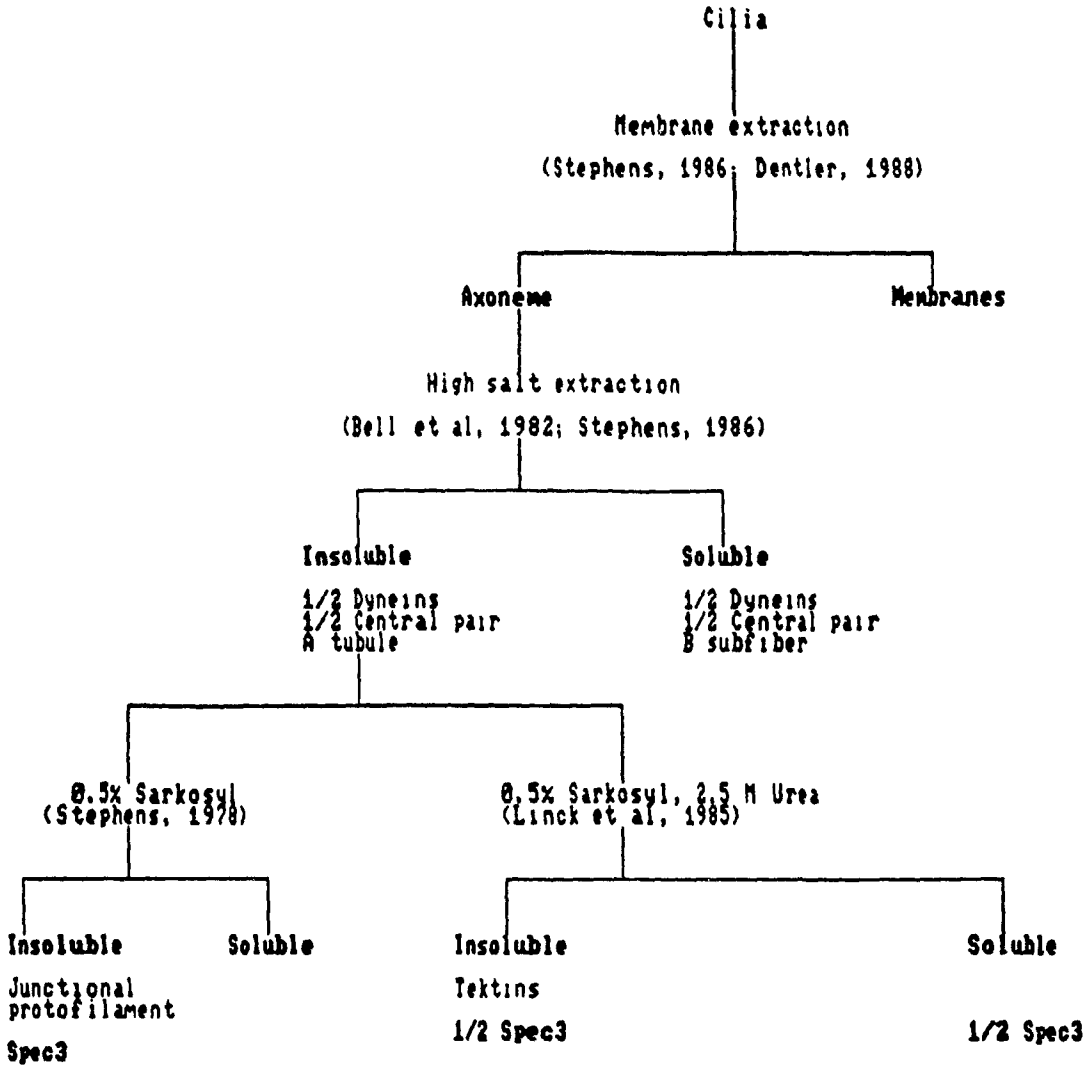


Fig. 19a illustrates the silver staining pattern of proteins from the various fractions separated by polyacrylamide gel electrophoresis. Stoichiometric amounts of cilia were loaded on each lane (except on lane 1 where one tenth the amount of solubilized whole cilia was loaded). Fig. 19b also compares different fractions on a mini gel.

The pattern of proteins in each fraction was different from the pattern described by Stephens (1986) for sperm flagella. Clearly, each step in the fractionation protocol removed different proteins Whether polypeptides extracted at each step correspond to what was expected cannot be proved For example, Triton X-100 or Triton X-114 should extract membrane proteins. Since there is no ciliary membrane marker for sea urchin embryos, I could not demonstrate that membranes were in fact solubilized by treatment with these two detergents Analysis by transmission electron microscopy on sections made at each fractionation step would help to elucidate this question

The abundance of tubulin in cilia is also an obstacle to the analysis of each fraction All lanes are overloaded with tubulin and underloaded with other proteins. In order to detect the less prevalent proteins, it was necessary to extend the silver staining reaction, resulting in a high background.

Stephens (1986) mentions that 0.6 M salt extraction should solubilize half of the dyneins from cilia. At about 1 cm from the top of Fig. 19a, one can see 2 or 3 protein bands that migrate similarly to dyneins. One of these bands was recovered in large amount in the membrane fraction (lane 2). The others separated in various quantities in all other fractions. In Fig 19b, because of the gel

system, it was not possible to identify dyneins. From these results it is difficult to determine the effectiveness of the fractionation procedure with respect to what could be expected from the literature. Analysis a the structural as well as biochemical level might resolve this question.

Tubulins and a band slightly larger than 45 kD (Fig 19a, arrowhead) were present in all fractions. Addition of 2.5 M urea to the Sarkosyl solution improved tubulin solubilization (Fig. 19b, lane 4). Linck et al. (1985) mention that by doing several rounds of Sarkosyl-urea extraction, they could solubilize most proteins except the tektins. With this gel system, it was difficult to determine clearly whether tektins remained insoluble.

DISCUSSION

The Spec3 antigen is associated with cilia of the oral and aboral ectoderm of the sea urchin embryo. It is different from other ciliary proteins in that it appears on cilia after ciliogenesis is completed and thus is not essential for ciliary movement. Its presence on cilia is correlated with a change in the pattern of movement of the blastula, from a disorganized, erratic movement to a spiral, directional movement, animal pole first.

Spec3 is also present in the Golgi complexes of cells of the aboral ectoderm. Experiments with monensin and the development profile of Spec3 suggest that it transits in the Golgi before being localized to cilia. Its absence in the Golgi of oral ectoderm cells could be due to a faster transport between the two compartments in the oral region of the embryo as compared to the aboral region. However, the absence of accumulation of Spec3 in the Golgi of oral cells after monensin treatment suggests that in that area, Spec3 does not transit in the Golgi before being localized to cilia.

Two different forms of the Spec3 antigen seem to coexist in the ectoderm. The Golgi or cytoplasmic antigen seems to have a higher affinity for the antiserum than the ciliary antigen and it does not compete well with the peptide against which the antiserum was raised. However, these two forms of the antigen cannot be distinguished on the basis of their mobility on SDS gels. The significance of the difference in antigenicity of the ciliary and the cytoplasmic form of Spec3 is not clear.

Although the predicted molecular weight of the Spec3 protein is about 21.6 kD, it migrates as a very large aggregate (MW > 350 kD) on

immunoblots. This aggregation is not due to cysteine cross-linking since reduction and alkylation does not disrupt it. Unexpectedly, the mobility on SDS gels of the Lytechinus pictus equivalent of Spec3 is closer to its predicted molecular weight (Eldon et al, 1989) even in the absence of reduction and alkylation. This difference in mobility could result from variations between the two sequences or from different interactions between Spec3 and other proteins in the two species.

The existence of a glycosylation site in the Spec3 sequence, its apparent large size and its presence in the Golgi apparatus suggest that it might be a glycoprotein. Inhibition of glycosylation with tunicamycin suggested a role for glycosylation of Spec3 but did not produce definitive results with immunocytochemical methods. If Spec3 is a glycoprotein, its mobility on SDS gels should be different in its glycosylated form from its non modified form. Proteins extracted from tunicamycin treated embryos could be compared to proteins from untreated embryos on immunoblots. The non-glycosylated form of Spec3 should migrate faster.

I have not been able to immunoprecipitate the Spec3 antigen although I have not tried this on isolated cilia. Since Spec3 is not identifiable on autoradiograms of ^{35}-S methionine labeled protein or on Coomassie blue stained gels, immunoprecipitation would be a useful tool in the study of this protein. If Spec3 is a glycoprotein, it should be identifiable as such by immunoprecipitation followed by carbohydrate staining or by radio-labelling with appropriate precursors. Immunoprecipitation would also allow detection of post-translational modifications such as phosphorylation.

Secretion from the Golgi apparatus seems to be essential for normal ciliary regeneration since monensin treatment inhibits it. It is not clear whether Spec3 or another protein is the limiting component for regeneration. Inhibition of glycosylation with tunicamycin prevents normal regeneration of cilia in Euglena (Bouk and Rogalski, 1982) but not in Tetrahymena (Keenan and Rice, 1980) It would be interesting to determine the effect of tunicamycin on cilia regeneration in sea urchin. The availability of a glycoprotein secreted by the Golgi might be necessary for normal regeneration

There is conflicting evidence regarding the precise localization of Spec3 in cilia. Its presence in the Golgi, the labeling of the ciliary surface by immunogold staining (Eldon et al, 1989) and the change in staining pattern caused by trypsin treatment suggest that it is a membrane or outer surface protein However, the absence of a signal peptide in the protein sequence (Eldon et al, 1989), the fractionation with the axoneme rather than with the membrane and matrix and the identity of staining of intact and demembranated isolated cilia militate for an axonemal protein Thus far, I cannot exclude that the Spec3 antigen is associated with a Golgi and a ciliary protein that are unrelated functionally However, results of monensin and puromycin treatments suggest that Spec3 transits from one compartment to the other and the indistinguishable electrophoretic properties of the two forms would be unlikely if they belong to different proteins.

The correlation between the appearance of Spec3 and the change in locomotory pattern suggests a causal relation between the two events. The fact that the beaded pattern of Spec3 appears at the same

events The fact that the beaded pattern of Spec3 appears at the same time as ciliary movement in regenerating cilia is consistent with that hypothesis Incorporation of the proline analogue in Spec3 might affect its function and result in aberrant movement of embryos Since it was not possible to extract all dyneins from ciliary axonemes, there is still a possibility that Spec3 is associated with the dynein arms, an association consistent with an involvment in motility.

It would be interesting to observe the motion of individual cilia before and after the appearance of Spec3. The "beak-like" projections in the lumen of the B-subfiber of doublets 1, 5 and 6 of Chlamydomonas flagella has been associated with the ability to execute a certain type of ciliary motion (Segal et al, 1984) Ultrastructural analysis by electron microscopy should reveal if such "beak-like" projections exist in urchin cilia and if their appearance is correlated with the appearance of Spec3. It would also be interesting to localize Spec3 in the axoneme by immunogold staining of isolated axonemes

The fact that Spec3 is absent from sperm flagella and endodermal cilia of the same species of urchins suggests a function specific to ectodermal cilia Such cilia are involved in movement, feeding behavior and avoidance of contact (Strathman, 1975, Mackie et al, 1969). Comparison of flagella, and endodermal and ectodermal cilia at the ultrastructural level might reveal significant differences that could be related to function

Allan and Kreis (1986) identified a membrane protein of the Golgi apparatus that is immunologically related to a brain microtubule associated protein. However, the coexistence of an

antigen in cilia and Golgi is unusual Mastigonemes, the hair-like projections of algal flagella, are assembled near the nuclear membrane and transit in the Golgi before being inserted at the base of the flagellum (Bouck, 1971). They are involved in locomotion and, in Chlamydomonas, in the agglutination of mating gametes. Like Spec3, they are insoluble in Sarkosyl but soluble in urea (Bouck, 1972) In a variety of flagellates, the surface of the flagellum is covered with small scales that might correspond to primitive mastigonemes (Cavalier-Smith, 1982)

Although I have not come across any mention of mastigonemes or ciliary scales in animals higher than sponges, it is interesting to speculate that Spec3 might be a component of a vestigial mastigoneme It would be consistent with its localization on the surface of cilia and in Golgi cisternae by immunogold staining (Eldon et al, 1989) and would accomodate the fact that it is not solubilized by detergent extraction and that it fractionates with the axoneme The beaded pattern of cilia could result from the insertion of this vestigial component in the ciliary membrane.

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