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EMAP, an echinoderm microtubule-associated protein found in microtubule-ribosome complexes

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SUMMARY

The major non-tubulin polypeptide found associated with microtubules purified from unfertilized sea urchin eggs by cycles of pH-dependent assembly has a M_r of 77,000. The 77,000 M_r polypeptide is heat- and acid-labile, and is antigenically distinct from the mammalian brain MAPs, MAP-2 and tau. Affinity-purified antiserum against the 77,000 M_r polypeptide was used to survey a variety of cells and tissues for the presence of antigenically related polypeptides. A cross-reacting polypeptide, ranging in M_r from 72,000 to 80,000, was found in microtubule preparations from a wide variety of echinoderms, including sea urchins, starfish and sand dollars. Indirect immunofluorescence showed that the polypeptide was found in interphase as well as mitotic microtubule arrays. No cross-reacting material was detected in microtubules isolated from marine molluscs, mammalian brain or mouse B16 cultured cells. Because the 77,000 M_r MAP is abundant in echinoderms, we have called it EMAP for echinoderm microtubule-associated protein. Although the precise function of the EMAP is not known, our data suggest that the EMAP is involved in the attachment of ribosomes to microtubules. Large numbers of ribosomes are attached to the walls of EMAP-containing microtubules, but not EMAP-deficient microtubules. Removal of the EMAP from the microtubule by salt-extraction results in the release of ribosomes from the microtubule, indicating that the EMAP may form part or all of the long tapered stalk that connects these two organelles.

Key words: microtubule-associated proteins (MAPs), *Lytechinus pictus*, *Strongylocentrotus purpuratus*, *Lytechinus variegatus*, *Arbacia punctulata*, *Echinarachnius parma*, *Asterias vulgaris*, sea urchins, starfish, sand dollars, oocytes, ribosomes

INTRODUCTION

Microtubule-associated proteins (MAPs) are the major accessory proteins on microtubules, the prominent, cylindrical component of the cytoskeleton. Despite the essential roles performed by microtubules in eukaryotic cells, little is known regarding the function of the filamentous MAPs, other that those that serve as motor proteins. Structural and biochemical analysis has revealed a great deal about the *in vitro* behavior of neuronal MAPs (Olmsted, 1986; Wiche et al., 1991). In addition, recent transfection experiments have suggested that brain MAPs promote microtubule assembly and stabilize microtubule arrays *in vivo* (Lewis et al., 1989; Kanai et al., 1989), as well as alter the morphogenesis of neuronal cells (Dinsmore and Solomon, 1991; Baas et al., 1991). Yet, the deletion of the DNA sequence encoding a 205,000 M_r MAP from *Drosophila* results in viable animals with no detectable phenotype (Pereira et al., 1992). These results indicate that we are only beginning to sort out the functional complexity of these MAPs.

Mammalian brain has been a major source of microtubule protein for the past 20 years because it contains large quantities of tubulin and MAPs that readily assemble into microtubules *in vitro*. However, the bulk of this microtubule protein originates from cells that have stopped dividing. To identify MAPs and other regulatory factors that may regulate microtubule function during mitosis, we have developed methods for the purification of tubulin (Suprenant and Rebhun, 1983) and microtubule protein (Suprenant and Marsh, 1987; Suprenant et al. 1989) from unfertilized sea urchin egg extracts in the absence of taxol (Vallee and Bloom, 1983; Bloom et al., 1985; Vallee and Collins, 1986; Scholey et al. 1984, 1985; Hirokawa and Hisanaga, 1987; Hosoya et al., 1990). The unfertilized sea urchin egg contains a large store of tubulin (Raff et al., 1971) that becomes incorporated into the first cleavage mitotic apparatus (Bibring and Baxandall, 1977) and ciliated blastula (Bibring and Baxandall, 1981). Although there are no detectable microtubules in unfertilized sea urchin eggs (Harris et al., 1980), microtubules readily assemble in cytosolic extracts prepared at alkaline pH (Suprenant and Marsh, 1987). When purified by further cycles of assembly and disassembly, these microtubules are found to be composed principally of tubulin and a 77,000 M_r polypeptide that coassembles stoichiometrically. Although this polypeptide has not been characterized, its abundance in these microtubule prepara-

tions suggests that it may be involved in the formation and function of the first mitotic apparatus.

To determine if the 77,000 M_r coassembling polypeptide is a MAP, we have prepared and characterized a monospecific antiserum against the SDS-denatured polypeptide and have identified its intracellular location in both embryonic and somatic cells. Using this reagent, we have found that the 77,000 M_r polypeptide is specifically associated with both interphase and mitotic microtubule arrays *in vivo*, but not with sperm flagellar microtubules. Moreover, this polypeptide may be involved in the interaction of the translational machinery with the cytoskeleton, as it appears to be involved in the binding of ribosomes to the microtubule walls. The abundance of this polypeptide in sand dollar, sea urchin and starfish eggs has led us to name this polypeptide EMAP for echinoderm microtubule-associated protein.

MATERIALS AND METHODS

Sea urchin, sand dollar, surf clam, and starfish eggs, oocytes and sperm

Surf clams (*Spisula solidissima*), starfish (*Asterias vulgaris*), sand dollars (*Echinarachnius parma*) and sea urchins (*Arbacia punctulata*) were provided by the Marine Resources Department at the Marine Biological Laboratory, Woods Hole, MA. The Pacific coast sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*), were obtained from Marinus, Inc., Long Beach, CA. Susan Decker (Hollywood, FL) provided the Gulf Coast sea urchin (*Lytechinus variegatus*). Eggs and sperm from the sea urchins (*S. purpuratus, A. punctulata, L. pictus, and L. variegatus*) and sand dollars (*E. parma*) were obtained by intracoelomic injection of 0.55 M KCl. Sperm were collected "dry" and stored on ice until needed. Surf clam (*S. solidissima*) and starfish (*A. vulgaris*) oocytes were obtained by teasing apart ovaries into Ca^{2+}-free artificial sea water (436 mM NaCl, 9 mM KCl, 34 mM $MgCl_2$, 16 mM $MgSO_4$, 1 mM EGTA, 5 mM Tris, pH 8.3). Eggs and oocytes were washed three times by settling through at least 20-30 volumes of Ca^{2+}-free artificial sea water. *S. purpuratus* eggs were dejellied by washing once in isotonic "19:1" (500 mM NaCl, 27 mM KCl, 2 mM EDTA, pH 7.8). All other eggs were dejellied by two to three additional washes in Ca^{2+}-free artificial sea water.

Sea urchin coelomocytes

Coelomocytes were isolated from the body cavity of the sea urchins *L. variegatus*, *L. pictus* and *S. purpuratus* exactly as described by Otto et al. (1979).

Bovine brain tissue

Microtubule protein was isolated from the brain cortex of freshly slaughtered beef cattle as described below for sea urchins.

Squid optic lobe

Squid (*Loligo pealei*) were obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA. Microtubules were prepared from freshly dissected optic lobes as described below.

Cultured mouse B16 melanoma cells

B16 cells were grown in monolayer in Dulbecco's MEM with glucose, glutamine and $NaHCO_3$, supplemented with 5% donor calf serum, at 37˚C and in 5% CO_2. Cells were washed once with phosphate-buffered saline (PBS) and detached from culture vessels by trypsinization (0.01% trypsin, 0.1% EDTA in PBS). Cells were harvested by centrifugation (5000 ***g***, 5 min) and washed twice in 1 mM PMSF in PBS. Microtubules were prepared as described below from the pelleted cells (6 g cells from 400 ml culture).

Microtubule protein

For most experiments, microtubule protein was purified from eggs, oocytes, neuronal tissue and mouse B16 cells by the pH and temperature-dependent assembly methods of Suprenant and Marsh (1987) as modified by Suprenant et al. (1989). This microtubule protein is referred to as "cycle-purified microtubule protein". For some experiments, microtubule protein was purified from unfertilized eggs by the taxol method (Vallee and Bloom, 1983; Vallee and Collins, 1986). In addition, cold-labile microtubule protein was obtained from mitotic apparatuses isolated as previously described from first cleavage embryos (Keller and Rebhun, 1981; Suprenant, 1986).

Measurement of protein concentration

Protein concentrations were determined by the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as a protein standard.

SDS-polyacrylamide gel electrophoresis and western blotting

Proteins were analyzed on SDS-urea-polyacrylamide gels with the discontinuous buffer formulation of Laemmli (1970) as previously described (Suprenant et al., 1989). For quantitation of the 77,000 M_r MAP, gels were stained with Fast Green (Gorovsky et al. 1970) and quantitated by densitometry.

For western blot analysis, unstained SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose (BA-45, Schleicher and Schuell, Keene, NH) with a semi-dry blot cell (Janssen Life Sciences, Piscataway, NJ) according to the manufacturers' instructions. After air-drying, the blots were blocked for 1 hour at room temperature in blocking buffer: Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 5% (w/v) Carnation nonfat dry milk. After blocking, the blots were incubated overnight at 4˚C with the affinity-purified rabbit antiserum against the 77,000 Mr sea urchin microtubule-associated protein (see below). The blots were washed four times (10 minutes each) in TBS and subsequently incubated for 2 hours at room temperature with a 1:3000 dilution (into blocking buffer) of the alkaline phosphatase (AP)-conjugated goat anti-rabbit secondary antibody (Zymed Laboratories, San Francisco, CA). The blots were washed four times in TBS and were developed in for 5-15 minutes in 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl2 containing 132 µl nitroblue tetrazolium (50 mg/ml in 70% dimethyl formamide) and 66 µl 5-bromo-4-chloro-3-indoly-phosphate (50 mg/ml in 100 % dimethyl formamide) (Blake et al., 1984).

Generation of polyclonal antiserum

Rabbit (New Zealand) pre-immune serum was collected and examined by immunofluorescence for the presence of autoantibodies that reacted with sea urchin (*S. purpuratus* embryos at first mitotic cleavage) or mammalian cytoskeletal proteins (mouse B16 cultured melanoma cells). Rabbits with preimmune serum against cytoskeletal proteins were not used. An immune serum was prepared against the 77,000 M_r polypeptide that coassembled with *S. purpuratus* egg microtubules. Microtubule protein was separated on a 3 mm thick preparative SDS-polyacrylamide gel (4% to 16% acrylamide and 1 M to 8 M urea gradient). The gel was stained briefly with 0.5% Coomassie blue R-250 in 40% methanol and 10% acetic acid and then destained in 25% methanol and 10% acetic acid. The band corresponding to the 77,000 M_r polypeptide was cut out and chopped into 2 mm square segments. The gel slices were washed

four times in distilled water (30 minutes each wash) and once in electro-dialysis buffer that contained 2.5 mM Tris, 19 mM glycine, and 0.1% (w/v) SDS. The protein was electroeluted from the gel slices at 200 V for 6 hours. The eluted protein was dialyzed against phosphate-buffered saline (PBS: 20 mM sodium phosphate, 0.13 M NaCl, pH 7.4). The dialyzed protein was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally. The rabbit was boosted subcutaneously on days 21, 28, 42 and 49 with dialyzed protein emulsified with incomplete adjuvant. Ten to fifteen ml of serum were collected 7 days after each boost, and once a month for the following year. Clotted serum was cleared by centrifugation (1000 g_{max}, 20 minutes, Beckman JA-20 rotor) and stored frozen at −20°C.

Blot-affinity purification of antiserum

The 77,000 M_r antiserum was mixed for 20 minutes on ice with an equal volume of ice-cold, saturated ammonium sulfate containing 10 mM K^+-EDTA, pH 7.0. The IgG-containing fraction precipitated and was collected by centrifugation at 14,000 revs/min for 20 minutes (JA-20 rotor). The pellet was washed three times by resuspension in saturated ammonium sulfate (above), followed by centrifugation. The pellet was resuspended in ice-cold PBS and dialyzed against several changes of PBS. The IgG fraction was affinity purified from nitrocellulose blots as described by Smith and Fisher (1984), concentrated with a Centricon 30 microconcentrater (Amincon Corp., Danvers, MA) and stored at 4°C.

Immunofluorescence light microscopy

S. purpuratus embryos were attached to poly(L-lysine)-coated glass coverslips, extracted in 25mM MES/K^+, pH 6.7, 10 mM EGTA, 0.5 mM $MgCl_2$, 25% glycerol, 25 mM PMSF and 1 % Nonidet P-40 (Balzcon and Schatten, 1981) and fixed at −20°C in 90% methanol containing 50 mM EGTA (Harris et al., 1980). Embryos were rehydrated in PBS and incubated for 30 minutes at 37°C with the affinity-purified anti-77,000 M_r antiserum. After five gentle washes with PBS, the coverslip-attached embryos were incubated for 30 minutes, at 37°C with a Texas Red-conjugated secondary antibody. Embryos were washed as before and mounted in 90% glycerol in 0.1 M Na_2HPO_4, pH 9.0.

Coelomocytes were permeabilized in methanol at −20°C, fixed in 1.5% formalin, and processed for immunofluorescence as described (Otto et al., 1979).

Sea urchin sperm were diluted into Ca^{2+}-free artificial sea water and adsorbed onto poly(L-lysine)-coated coverglass. Sperm were fixed at −20°C with methanol-EGTA and processed for immunofluorescence.

Electron microscopy

Microtubule pellets were fixed and embedded for thin-sectioning as previously described (Suprenant and Marsh, 1987).

RESULTS

A 77,000 M_r polypeptide coassembles with sea urchin microtubules assembled at alkaline pH

In this study, microtubules were purified from unfertilized sea urchin egg extracts by cycles of pH- and temperature-dependent assembly (Suprenant and Marsh, 1987; Suprenant et al., 1989). The major non-tubulin component of these microtubules is a 77,000 M_r polypeptide (Fig. 1A). Several other higher and lower molecular weight polypeptides are present at reduced levels. Filamentous projections, similar to those produced by the mammalian brain MAPs, are not found on sea urchin microtubules assembled *in vitro*

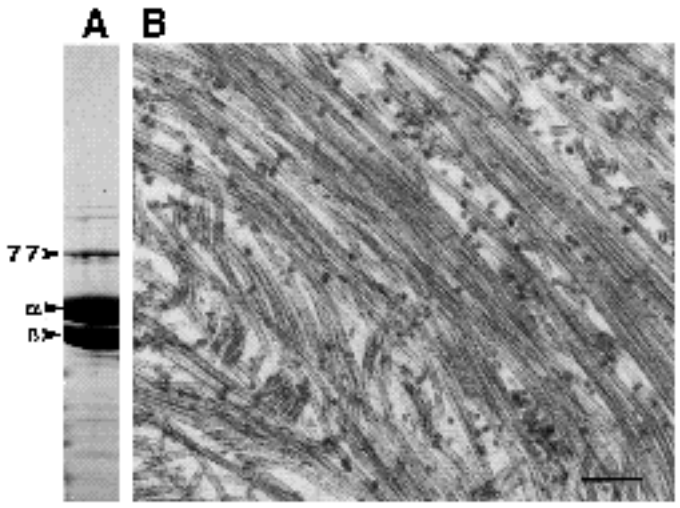

Fig. 1. Purification of microtubules from unfertilized sea urchin (*Strongylocentrotus purpuratus*) eggs. (A) SDS-PAGE gel shows that a 77,000 M_r polypeptide and α and β tubulin are the major microtubule proteins. (B) Electron micrograph of egg microtubules with associated ribosomes. Bar, 200 nm.

(Fig. 1B). In contrast, microtubules are decorated with large globular structures, previously identified as ribosomes (Suprenant et al., 1989). In this previous study, we demonstrated that the ribosomes are attached to microtubules by ionic interactions through a long tapered stalk. A potential candidate for this stalk is the 77,000 M_r polypeptide (and presumptive MAP). In addition to interacting with the translational machinery, the 77,000 M_r polypeptide may regulate microtubule dynamics during the embryonic cell cycles. To begin to understand the function of this protein, we have examined its abundance, its conservation among echinoderms and its intracellular location in embryonic and differentiated cells.

The 77,000 M_r polypeptide is a major component of microtubule preparations from a wide variety of sea urchins including cold water (*S. purpuratus*, *L. pictus*) and warm water species (*L. variegatus*, *A. punctulata*). Among the four different species of urchins examined, the "77,000 M_r" polypeptide varied both in its relative abundance and its migration on SDS-PAGE. Consistently, the largest amounts of this polypeptide were found in microtubules isolated from the warm water urchins. The stoichiometric extremes are illustrated in Fig. 2. For quantitation, purified microtubule protein from *S. purpuratus* and *A. punctulata* were compared side-by-side on Fast green-stained acrylamide gels. Densitometric analysis of these gels indicates that a 75,000 M_r and a 77,000 M_r polypeptide comprises 17% by mass of the *A. punctulata* microtubules and 6% by mass of the *S. purpuratus* microtubules, respectively. The molar ratio of the 75,000 M_r and 77,000 M_r polypeptides to tubulin dimer is 0.22±0.01 and 0.08 ± 0.02 for *A. punctulata* and *S. purpuratus* microtubules, respectively.

Generation of a monospecific antiserum against the 77,000 M_r polypeptide

Attempts to purify sufficient quantities of EMAP for bio-

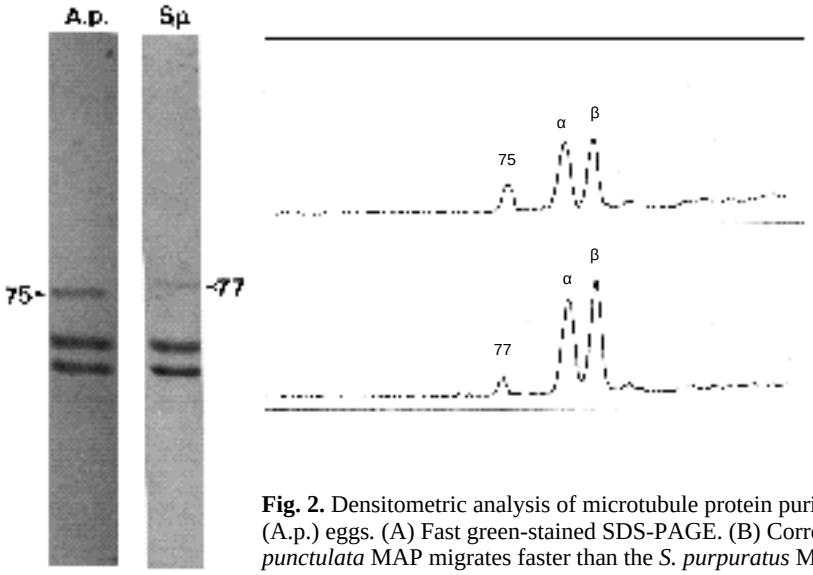

Fig. 2. Densitometric analysis of microtubule protein purified from *S. purpuratus* (S.p.) and *A. punctulata* (A.p.) eggs. (A) Fast green-stained SDS-PAGE. (B) Corresponding densitometric tracings. Note that the *A. punctulata* MAP migrates faster than the *S. purpuratus* MAP. M_r shown $\times 10^{-3}$.

chemical analysis have been largely unsuccessful. EMAP binds weakly to cation-exchange columns such as carboxymethylcellulose, traditionally used to purify MAPs (Weingarten et al., 1975; Williams and Detrich, 1979). Because of difficulties in the purification of this polypeptide, an immunological approach was utilized for the initial characterization. The 77,000 M_r polypeptide from *S. purpuratus* cycle-purified microtubule preparations was used as an immunogen in rabbits. The polyclonal antiserum generated against the SDS-denatured 77,000 M_r band recognizes the 77,000 M_r polypeptide on western blots (Fig. 3A-C). This antiserum is highly reactive and shows significant binding to the 77,000 M_r polypeptide at dilutions of 1:10,000 and greater. In addition, the antibody recognizes native protein that has been blotted directly to nitrocellulose (data not shown). A monospecific IgG fraction was purified from the crude antiserum by ammonium sulfate precipitation and affinity-purification from nitrocellulose blots containing the 77,000 M_r polypeptide (Smith and Fisher, 1984). The affinity-purified anti-77,000 M_r IgG recognizes a single polypeptide band of M_r 77,000 in whole egg homogenates (Fig. 3D,E); however, the 77,000 M_r polypeptide is not detectable in flagellar axonemes (Fig. 3F,G).

Microtubules in embryonic and somatic cells are stained with the 77,000 M_r antiserum

First cleavage embryos were probed with the affinity-purified anti-77,000 M_r antibodies (Fig. 4). The mitotic apparatus is prominently labeled with this antibody and the staining pattern is frequently along linear tracks presumed to be microtubules. Immunostaining was distributed throughout the spindle, although astral regions of the spindle appear to be stained more intensely than the central spindle regions. The antibody stains the mitotic spindle apparatus of all the sea urchins examined in this study.

To compare the localization of the 77,000 M_r polypeptide in mitotic cells with that in interphase cells, we examined sea urchin coelomocytes. The coelom or body cavity of the adult sea urchin contains several different types of coelomocytes (Edds, 1985; Smith, 1981). One coelomocyte, the phagocytic amoebocyte, can be purified in large numbers from the coelomic fluid and will adhere and spread on a glass coverslip into their characteristic petaloid shape (Fig. 5A). These adult somatic cells are terminally differ-

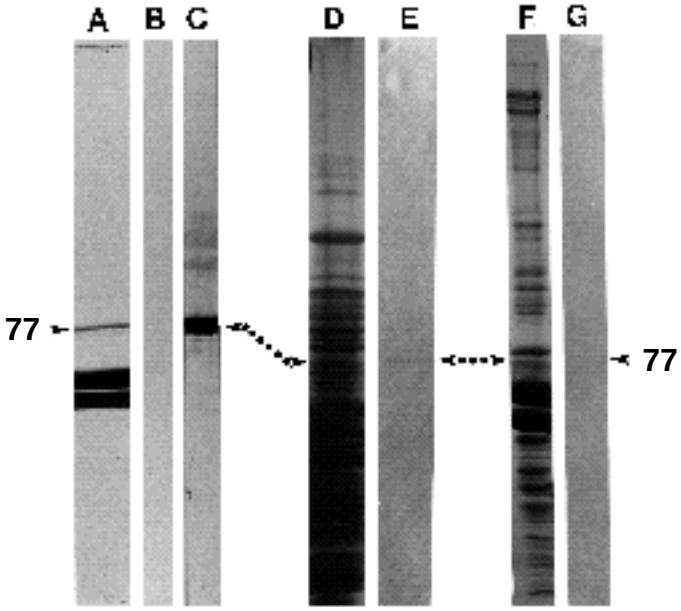

Fig. 3. Immunoblot analysis of the polyclonal antibody generated against the 77,000 M_r polypeptide. (A) Electrophoretic analysis of *S. purpuratus* egg microtubule protein stained with Coomassie blue. Corresponding immunoblot with preimmune serum (B) and unfractionated anti-77,000 M_r antiserum (C). (D) Proteins in a whole-egg homogenate separated by SDS-PAGE and corresponding immunoblot with the affinity-purified anti-77,000 M_r IgG. (F) Electrophoretic separation of axonemal proteins from sea urchin sperm and corresponding immunoblot with the affinity-purified anti-77,000 M_r IgG (G). The 77,000 M_r polypeptide is detectable in eggs but not sperm. Arrowheads and dotted lines indicate the location of the 77,000 M_r polypeptide on gels of different proportion.

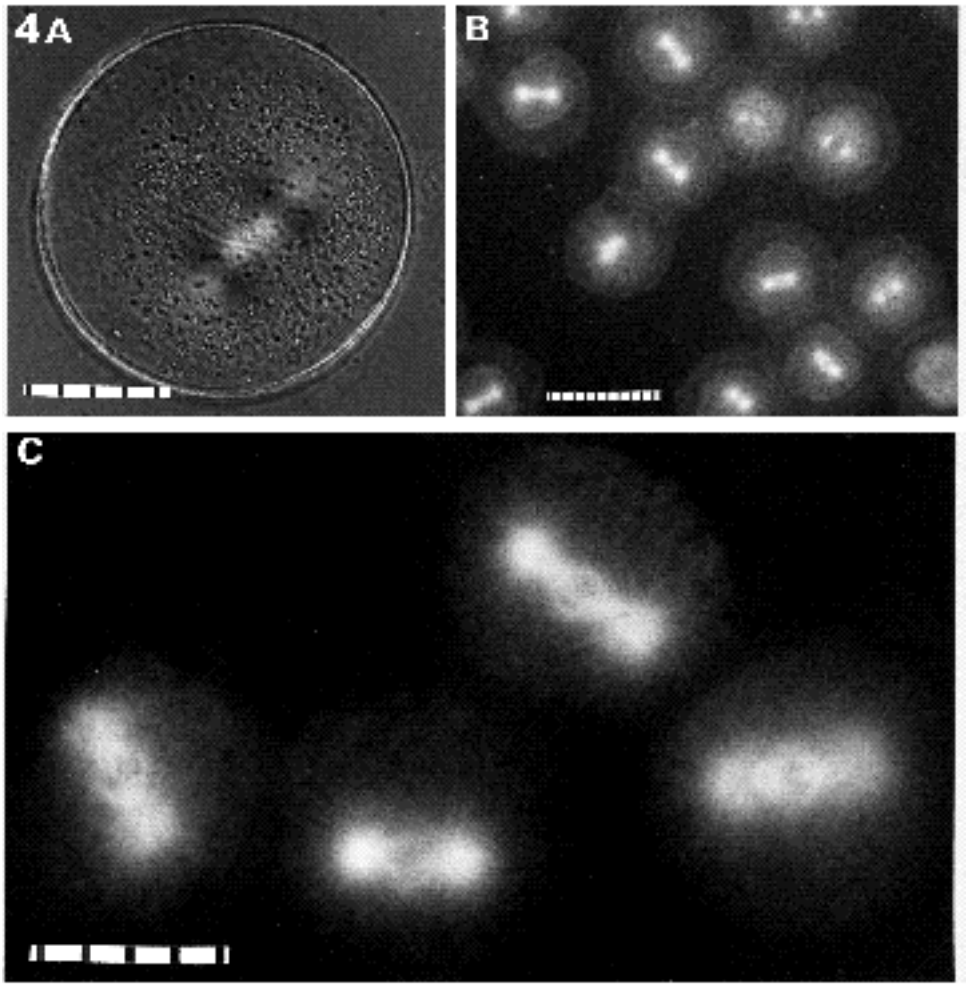

Fig. 4. Immunofluorescence staining of first cleavage sea urchin embryos. (A) Extracted embryo viewed in polarized light prior to immunofluorescence. (B, C) Staining of *S. purpuratus* mitotic apparatus with the affinity-purified anti-77,000 M_r IgG. The 77,000 M_r polypeptide is detectable in spindles at all stages of mitosis. Bar, 10 μm spacing.

entiated and do not divide. Because these cells contain fewer microtubules than the first cleavage embryos and their interphase microtubule array is flattened and nearly two-dimensional, these cells are ideal for immunolocalization studies. In addition, coelomocytes can be prepared for immunofluorescence without detergent extraction, a procedure that can sometimes cause the redistribution of soluble antigens (Melan and Sluder, 1992).

Fig. 5B-E illustrates the petaloid coelomocyte staining pattern with DM1A, a monoclonal antibody against α tubulin, and with the affinity-purified anti-77,000 M_r IgG. Individual microtubules can be visualized by immunofluorescence staining with the DM1A anti-tubulin antibody. The coelomocyte microtubules appear to originate near the nucleus and extend outwards toward the cell periphery. The affinity-purified anti-77,000 M_r IgG also stains the coelomocytes in a pattern similar to that of the DM1A tubulin staining. Curvilinear tracks, presumed to be microtubules extend radially outward from the nucleus. The staining pattern becomes diffuse and barely detectable when the cells are treated with nocodazole (data not shown). These results indicate that the 77,000 M_r polypeptide is microtubule-associated. It is interesting that both the anti-tubulin and anti-77,000 M_r staining patterns are perinuclear, not pericentriolar. There is no obvious focal nucleation center in these cells at any plane of focus.

The 77,000 M_r MAP is detectable only on cytoplasmic or mitotic microtubule arrays. Immunofluorescence microscopy failed to reveal any cross-reacting structure in intact sperm (Fig. 6). Although the sperm tail stains brightly with the anti-tubulin antibody, there was no detectable staining of the flagellum with the anti-77,000 M_r IgG. This confirms the western blot results shown in Fig. 3.

The 77,000 M_r MAP is present in mitotic apparatus microtubules and in taxol-polymerized microtubules

Several MAPs of similar M_r have been identified in microtubule preparations from sea urchin eggs. Keller and Rebhun (1982) identified an 80,000 M_r MAP from *S. purpuratus* mitotic apparatuses that copurified with spindle tubulin. Bloom and coworkers used a taxol-dependent procedure to purify microtubules from unfertilized *L. variegatus* and *S. purpuratus* eggs and identified four MAPs of M_r 205,000, 120,000, 100,000 and 77,000 (Vallee and Bloom, 1983; Bloom et al. 1985). In a similar report, an 80,000 M_r MAP was identified in taxol-microtubule preparations from *S. purpuratus*, *L. pictus* and *S. droebachiensis* (Scholey et al., 1984). Finally, Hirokawa and Hisanaga (1987) used gel filtration chromatography to purify a 75,000 M_r MAP from taxol-microtubules isolated from unfertilized eggs of the sea

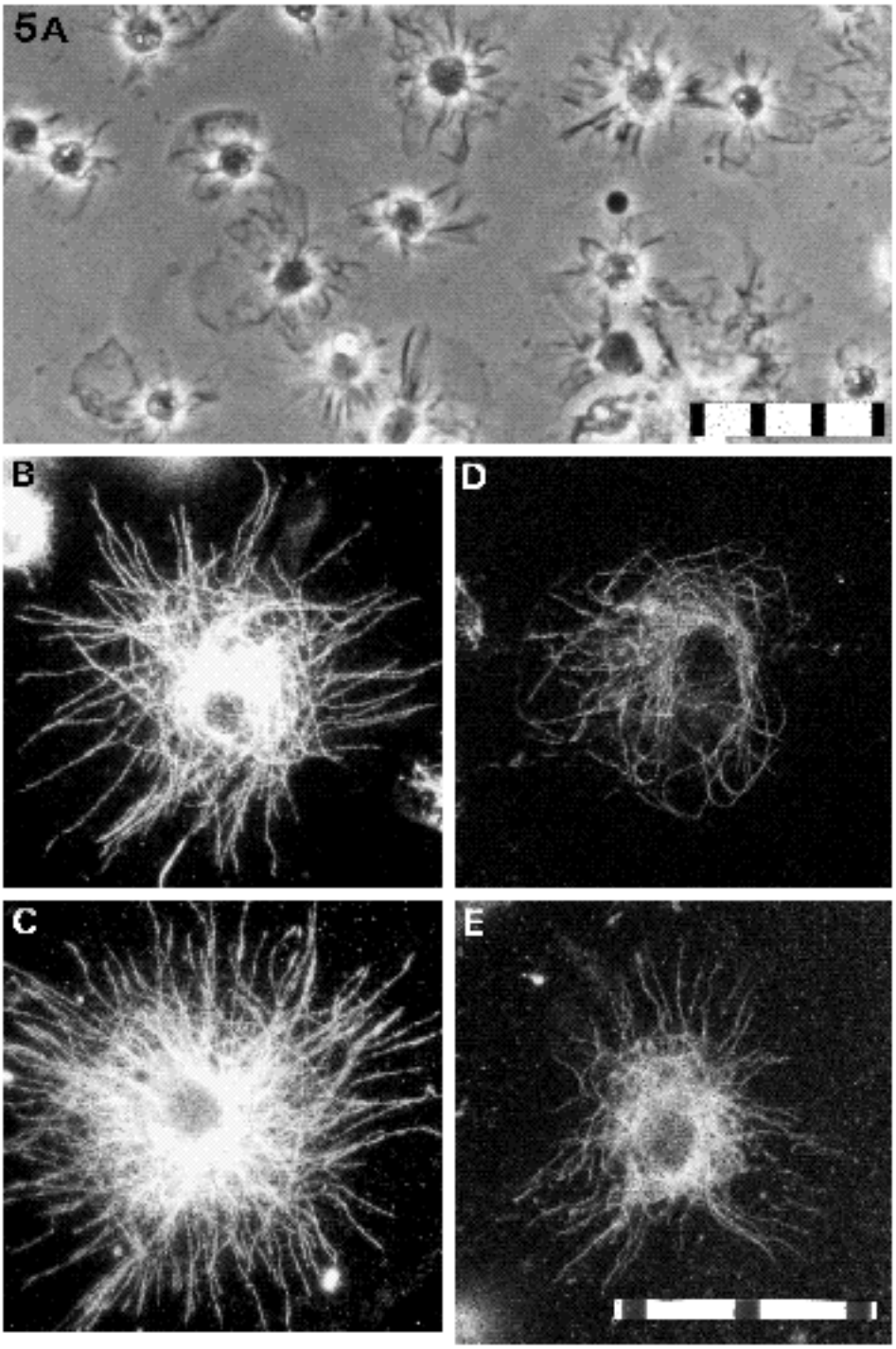

Fig. 5. Immunofluorescence microscopy of *L. variegatus* coelomocytes. (A) Phase-contrast micrograph of petaloid coelomocytes purified from the sea urchin *L. variegatus*. Coelomocyte staining pattern with DM1A anti-tubulin antibody (B,C) or the affinity-purified anti-77,000 M_r IgG (D,E). A different coelomocyte is shown in each panel. Similar staining patterns were observed in coelomocytes isolated from *L. pictus* and *S. purpuratus*. Bar, 10 μm spacing.

urchins *Pseudocentrotus depressus* and *Hemicentrotus pul - cherrimus*.

To determine whether the 75,000 to 80,000 M_r taxol MAPs (Vallee and Bloom, 1983; Bloom et al., 1985; Scholey et al., 1984; Hirokawa and Hisanaga, 1987) and the 80,000 M_r spindle MAP (Keller and Rebhun, 1980; Rebhun et al., 1982) are antigenically related to the 77,000 M_r cycle-purified MAP, these microtubule proteins were isolated and blotted with the anti-77,000 M_r antiserum. On a urea and acrylamide gradient gel, a complex pattern of polypeptides is observed in the 77,000 M_r region of the taxol-microtubule preparation (Fig. 7a,b). Three bands with M_r values of 78,000, 80,000 and 82,000, are observed in *L. pictus* microtubule preparations. The affinity-purified anti-77,000 M_r IgG recognizes a single band that corresponds to the most abundant polypeptide migrating with an apparent M_r of 80,000 (Fig. 7a′,b′). The *L. pictus* MAP migrates with a slightly larger M_r than the *S. purpuratus* MAP regardless of whether it was taxol- or cycle-purified.

In a separate experiment, *L. pictus* eggs were fertilized and allowed to develop to the first mitotic metaphase. Mitotic apparatuses were isolated as previously described

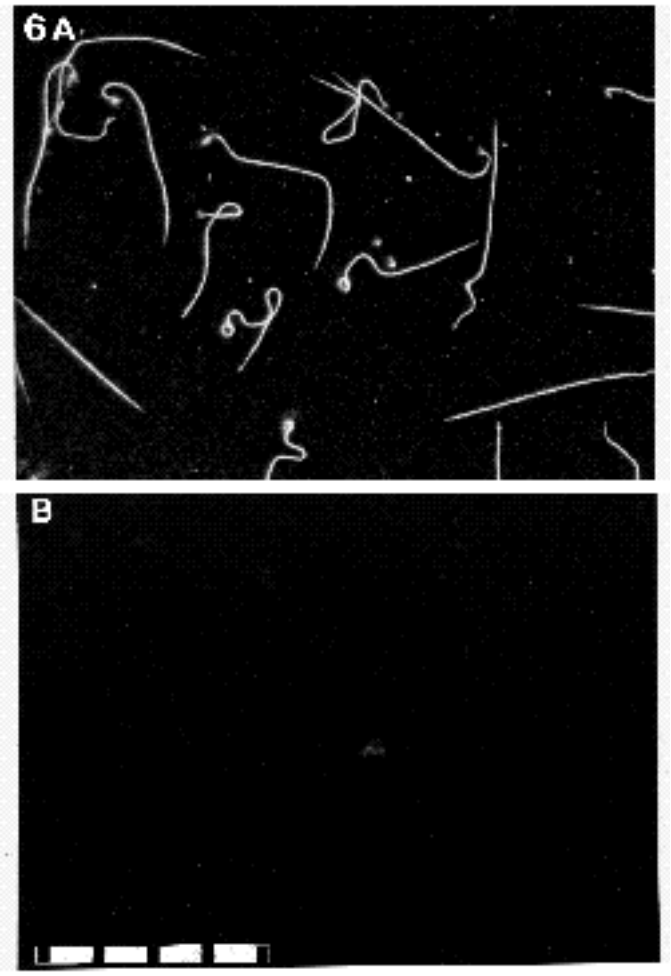

Fig. 6. Immunofluorescence microscopy of *S. purpuratus* sperm with the DM1A anti-tubulin antibody (A) and the affinity-purified anti-77,000 M_r IgG (B). The 77,000 M_r polypeptide is not detectable in sea urchin sperm shown in B. Bar, 10 μm spacing.

Table 1. Presence or absence of the 77,000 M_r MAP in several species

	Presence	Absence
Phylum Echinodermata		
Sea urchin eggs and coelomocytes		
Strongylocentrotus purpuratus	+	
Lytechinus pictus	+	
Lytechinus variegatus	+	
Arbacia punctulata	+	
Sea urchin sperm flagella		
Strongylocentrotus purpuratus		+
Lytechinus pictus		+
Starfish oocytes		
Asterias vulgaris	+	
Sand dollar eggs		
Echinarachnius parma	+	
Phylum Mollusca		
Clam oocytes		
Spisula solidissima		+
Squid optic lobe		
Loligo pealei		+
Subphylum Vertebrata		
Brain microtubule protein		
Bos tauris		+
B16 cultured melanoma cells		
Mus musculus		+

Several species of animals were examined by western blotting and immunofluorescence techniques for the presence of the 77,000 M_r MAP. The abundance of this polypeptide in sand dollar, sea urchin and starfish has led us to name this polypeptide EMAP for echinoderm microtubule-associated protein.

(Keller and Rebhun, 1981; Suprenant, 1986) and placed on ice to depolymerize the mitotic microtubules. After centrifugation, a cold-labile microtubule fraction was separated from the insoluble spindle remnant. Proteins from these two fractions were separated by electrophoresis and immunoblotted with the affinity-purified anti-77,000 M_r IgG (Fig. 7c,d,e). As can be seen on the Coomassie blue-stained gel, most of the tubulin and all of the detectable 80,000 M_r MAP is solubilized in the cold. Only traces of 80,000 M_r MAP are found in the spindle remnant. Again, the anti-77,000 M_r IgG recognizes a single polypeptide in the soluble microtubule fraction that corresponds to the previously identified 80,000 M_r spindle MAP (Fig. 7c′,d′,e′).

The 77,000 M_r polypeptide is abundant in echinoderm microtubule preparations

The 77,000 M_r MAP is not restricted to sea urchin eggs and coelomocytes. Microtubules were purified from a variety of marine eggs and tissues by cycles of pH- and temperature-dependent assembly, and immunoblotted with the affinity-purified anti-77,000 M_r antibody (Fig. 8). Microtubule preparations from starfish oocytes contain large quantities of a 72,000 M_r polypeptide that is antigenically related to the 77,000 M_r sea urchin MAP (Fig. 8b,b′). Microtubules were also purified from *E. parma*, an Atlantic coast sand dollar. On Coomassie blue-stained gels there are few detectable MAPs in the sand dollar preparation; however, an immunoblot of the same microtubule protein identifies two proteins of M_r 80,000 and 100,000 that react strongly with the anti-77,000 M_r antibody (Fig. 8f,f′). In contrast, no cross-reacting material was detected in microtubule preparations from marine molluscs, including surf clam oocytes (*S. solidissima*) and squid optic lobe (*L. pealei*); although other putative MAPs are present in these preparations. Similarly, no antigenically related polypeptides were detected in bovine brain microtubule preparations or in mouse B16 cultured melanoma cells. These results are summarized in Table 1.

EMAP is distinct from MAP-2 and tau

EMAP and the vertebrate brain MAPs appear to be antigenically distinct. The antiserum against the EMAP fails to react with any MAP in bovine brain microtubule preparations (Fig. 8e,e′). In addition, monoclonal antibodies against MAP-2 or tau (tau-1 and AP-14, kindly provided by L. Binder, University of Alabama, Birmingham) fail to recognize the 77,000 M_r sea urchin MAP (data not shown). Unlike vertebrate MAPs, EMAP is not heat-stabile. *S. purpuratus* microtubule protein was resuspended in ice-cold buffer containing 100 mM PIPES/K^+ (pH 6.9), 1 mM EGTA, 1 mM $MgSO_4$, 2 mM DTT, 1 mM GTP, 0.2 mM PMSF, 3 μg/ml leupeptin and 10 μg/ml pepstatin. After a

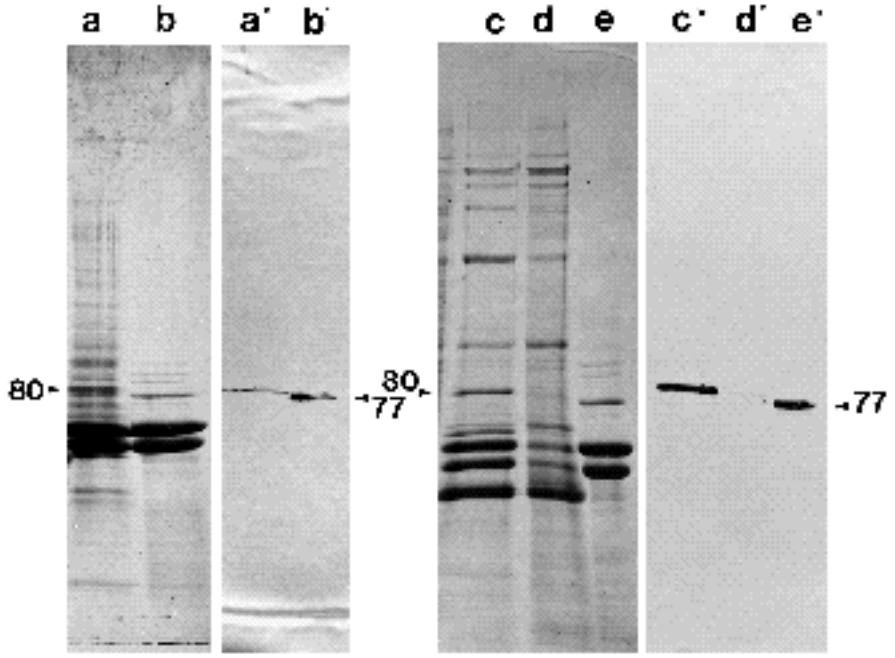

Fig. 7. Evidence that the taxol MAP and 80,000 M_r spindle MAP from *L. pictus* are antigenically related to the 77,000 M_r cycle-purified MAP. SDS-PAGE of taxol microtubules from *L. pictus* (a) and cycle-purified microtubules from *S. purpuratus* (b) and their corresponding immunoblot with the affinity-purified anti-77,000 M_r IgG (a′,b′). The corresponding MAP in *L. pictus* migrates with an apparent M_r of 80,000. To identify the 80,000 M_r spindle MAP, mitotic apparatuses were isolated from *L. pictus* embryos and placed on ice to depolymerize microtubules. After a brief centrifugation the cold-labile microtubule protein (c) was separated from the insoluble spindle remnants (d). The protein composition of *S. purpuratus* cycle-purified microtubule protein is compared with this protein on this Coomassie blue-stained SDS-PAGE gel (e). The corresponding immunoblot with the affinity-purified anti-77,000 M_r IgG is shown on the right (c′,d′,e′). The position of the 77,000 M_r cycle-purified MAP from *S. purpuratus* is shown. The corresponding MAP in *L. pictus* mitotic apparatuses also migrates with a higher apparent M_r of 80,000.

30-minute incubation on ice to depolymerize the microtubules completely, the solution was immersed into a 100°C water bath for 5 minutes. After centrifugation (39,000 ***g***, 30 minutes, 4°C), the soluble supernatant fraction and resuspended pellets were analyzed by SDS-PAGE. All the detectable 77,000 M_r polypeptide is in the heat-denatured pellet fraction. Finally, unlike mammalian tau protein (Lindwall and Cole, 1984), the 77,000 M_r sea urchin MAP is completely insoluble in 2.5% perchloric acid (data not shown). These results indicate that EMAP may belong to a unique class of microtubule-associated proteins distinct from the vertebrate brain MAPs.

EMAP may be involved in ribosome attachment to microtubules

There is a strong correlation between the presence of the 77,000 M_r EMAP and the interaction of ribosomes with microtubules assembled *in vitro*. Electron microscopy reveals that ribosomes are absent in microtubule preparations where the EMAP is not detectable (Fig. 9). In contrast, ribosomes are abundant in EMAP-containing microtubules. These results suggest that ribosomes do not randomly bind to microtubules assembled under our buffer conditions, but rather they associate with a specific MAP-containing microtubule.

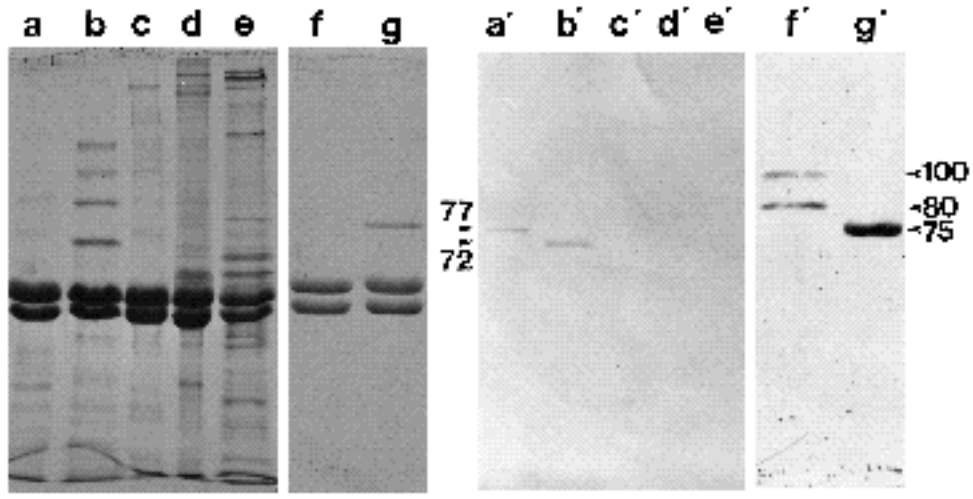

Fig. 8. Starfish and sand dollar MAPs are antigenically related to the 77,000 M_r sea urchin MAP. Microtubule protein was purified from sea urchin eggs (*S. purpuratus*, a; *A. punctulata*, g), starfish oocytes (b), surf clam oocytes (c), squid optic lobes (d), bovine brain (e), and sand dollar eggs (f), and analyzed by SDS-PAGE. Their corresponding immunoblots with the affinity-purified anti-77,000 M_r IgG are shown in a′-g′. The antibody recognizes a 72,000 M_r starfish MAP and 75,000 M_r and 77,000 M_r sea urchin MAP; however in sand dollars, two polypeptides of M_r 80,000 and 100,000 are detected. There is no cross-reacting polypeptide detected in surf clam, squid or mammalian brain microtubules.

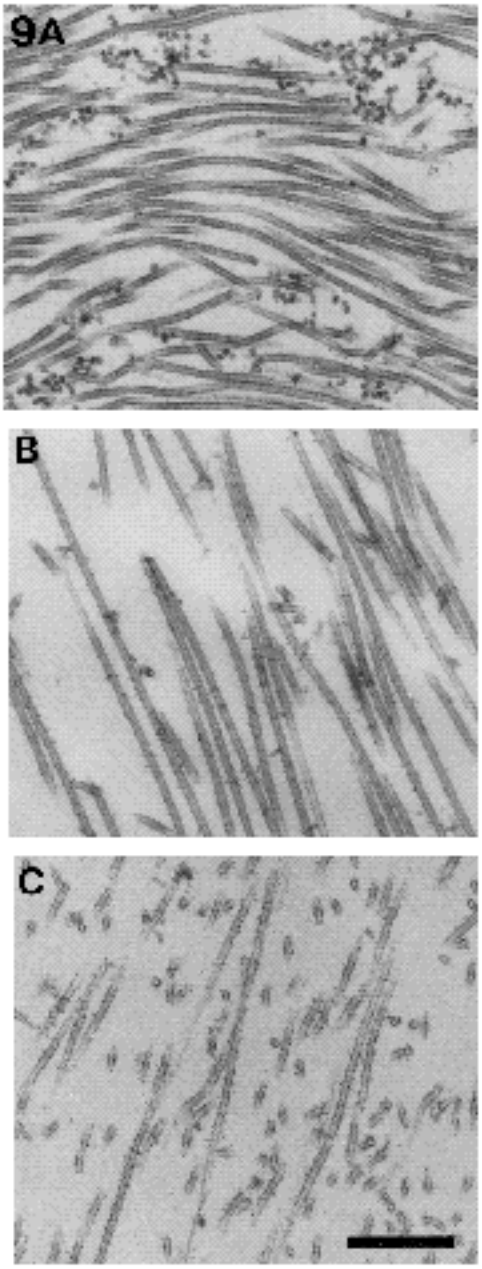

Fig. 9. Ribosomes are abundant in sea urchin (*A. punctulata*) microtubule preparations (A). These microtubules were pelleted, fixed and thin-sectioned for electron microscopy. Thre are no detectable ribosomes in identically prepared microtubules from surf clams (B) and mammalian brain (C). In contrast, these microtubules are decorated with fuzzy, filamentous projections. Bar, 400 nm.

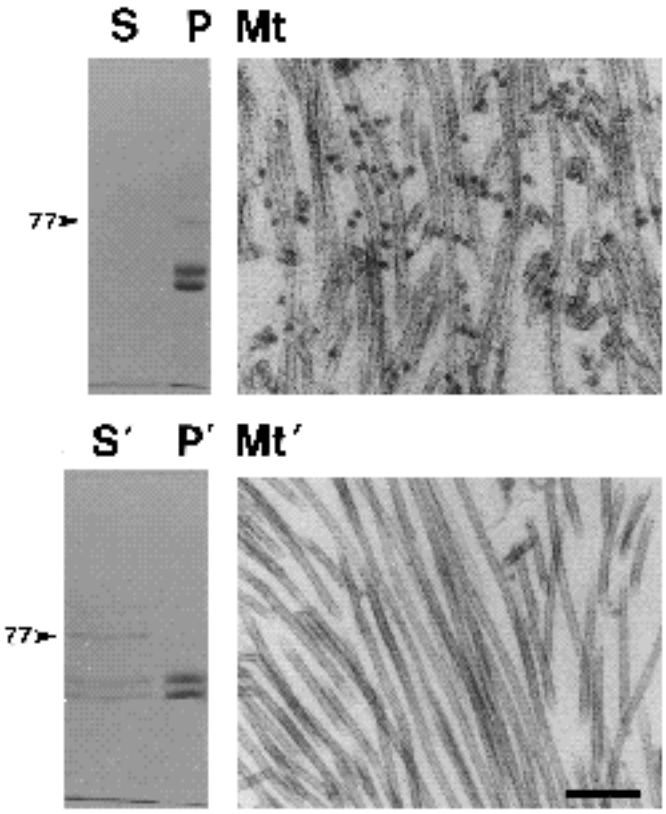

Fig. 10. Salt-extraction of the EMAP also results in the loss of microtubule-associated ribosomes. Sea urchin microtubules (*S. purpuratus*) were assembled in the presence of 20 μM taxol and 1 mM GTP for 3 min at 37°C, pelleted at 18,000 revs/min (JA20) for 30 minutes and subsequently analyzed by SDS-PAGE and electron microscopy. There are no detectable microtubule proteins in the 18K supernatant (S), while tubulin and the 77,000 M_r EMAP are the major microtubule proteins found in the pellet (P). Electron microscopy reveals that these microtubules (Mt) are decorated with many darkly stained ribosomes. In contrast, when sea urchin microtubules are polymerized in the presence of taxol, GTP and 0.35 M NaCl, the 77,000 M_r EMAP is found in the supernatant (S′) and is not detected in the pellet (P′). These microtubules (MT′) are also strikingly devoid of ribosomes. Bar, 200 nm.

Removal of the EMAP results in the loss of microtubule-associated ribosomes. EMAP cosediments with sea urchin egg microtubules that are purified by three cycles of pH- and temperature-dependent assembly and subsequently stabilized with taxol. Washing these taxol-stabilized microtubules with microtubule assembly buffer fails to remove any appreciable quantity of the EMAP and numerous ribosomes remain attached along the surface of these pelleted microtubules (Fig. 10). However, subsequent washing of the egg microtubles with assembly buffer containing 0.35 M NaCl results in the complete removal of the EMAP and the associated ribosomes. These results suggest that ribosome-microtubule interactions may be mediated through the EMAP.

DISCUSSION

In this report, we establish the identity of an echinoderm microtubule-associated protein or EMAP. EMAP is identified as a microtubule-associated protein because it copurifies with microtubules isolated from sea urchins, starfish and sand dollars, and a monospecific antibody against the EMAP localizes to microtubules in the mitotic apparatus and the cytoplasmic microtubule complex in interphase cells. The EMAP is not restricted to microtubules during a

particular stage of the cell cycle nor is it restricted to a particular cell type, as it is found in oocytes, eggs and adult coelomocytes. EMAP is restricted, however, to echinoderms. Within the Phylum Echinodermata, the EMAP was detected in oocytes prior to meiosis and in mature eggs, in both embryonic and adult cells, and on mitotic as well as interphase microtubule arrays. EMAP was abundant in the Class Asteroidea (starfish) and both "Regularia" (sea urchin) and "Irregularia" (sand dollar) Echinodea subclasses. EMAP was not detected, however, in embryonic or somatic cells of marine molluscs from the Class Bivalvia (surf clam) or the Class Cephalopoda (squid). In addition, EMAP cross-reactivity was not found in bovine brain microtubule preparations or in mouse B16 cultured melanoma cells.

Biochemical studies indicate that EMAP is a unique polypeptide, perhaps unrelated to the vertebrate MAPs. EMAP is not thermo- or acid-stable, nor does it cross-react with antibodies against MAP-2 and tau. The EMAP antiserum does not recognize any bovine brain MAPs. However, these results do not preclude the possibility that there is a functional EMAP homologue in these organisms; the antiserum may recognize only the most evolutionarily related polypeptides. Future molecular studies will determine whether EMAP shares any sequence or perhaps functional homology with the more extensively characterized MAPs.

In this paper, we have shown that EMAP is antigenically related to the previously identified 75-80,000 M_r taxol MAP (Vallee and Bloom, 1983; Bloom et al., 1985; Scholey et al., 1984; Hirokawa and Hisanaga, 1987; Hosoya et al., 1990) and 80,000 M_r spindle MAP (Keller and Rebhun, 1982; Rebhun et al., 1982). The antiserum against the 77,000 M_r cycle-purified MAP reacts strongly and specifically with both the taxol and spindle MAP. Moreover, a monoclonal antibody, S.P. 77-2, prepared against the 77,000 M_r taxol MAP, reacts strongly with the EMAP on western blots (antibody kindly provided by R. Vallee, Worcester Foundation, Worcester, MA) (data not shown). Both the polyclonal antibody against the cycle-purified MAP and the monoclonal antibody against the taxol-MAP stain microtubules of the mitotic spindle apparatus and phagocytic amoebocyte (this report, and Bloom et al., 1985). In addition, both antisera fail to recognize any antigenically related polypeptide in sperm flagella. From these results, it is likely that the taxol-, spindle- and cycle-purified MAPs are the same protein.

Prior to this report, the relationship among the various sea urchin MAPs had not been established. It was premature to assume that they might be the same molecule because it was shown previously that mitotic cytoskeletons isolated from the spiny red urchin, *Strongylocentrotus franciscanus*, contained several proteins that migrated from 74,000 to 80,000 M_r on 2-D SDS-polyacrylamide gels (Leslie and Wilson, 1989). This raised the possibility that there were several different MAPs in this M_r range with potentially different functions. On high-resolution SDS-urea-acrylamide gels, there are frequently two to three bands in the 75,000 to 82,000 M_r range, particularly in taxol-microtubule preparations (see Fig. 7). This heterogeneity is not restricted to taxol-microtubule preparations. Frequently, there are two bands of similar M_r in the cycle-purified preparations (see Fig. 1). With the exception of sand dollar microtubules, only a single, major polypeptide reacts with the EMAP antiserum. It was suggested in a brief report (Scholey et al., 1985), that a minor 78,000 M_r polypeptide in the taxol-microtubule preparations is a low-affinity microtubule-binding protein. Immunolocalization studies suggest that the 78,000 M_r antigen is mitotic spindle-associated, but not necessarily microtubule-associated *in vivo*. Understanding the function of these polypeptides, and their relationship to the EMAP, awaits further study.

What is the function of the EMAP? Since the immunolocalization studies indicate that the EMAP is present on both cytoplasmic and spindle microtubules, it is unlikely that the EMAP functions only in mitosis. However, recent studies indicate that microtubule dynamics during the cell cycle may be controlled through the actions of the cdc2 (Verde et al., 1990; Faruki et al., 1992) and mitogen-activated protein kinases (Gotoh et al., 1991; Hoshi et al., 1992). Although the *in vivo* targets for these enzymes are unknown, it is possible that the EMAP could be phosphorylated by one of these pathways and subsequently bring about a cell cycle-dependent change in microtubule dynamics.

Another potential function for the EMAP is in the association of ribosomes with the cytoskeleton. Previously, work from our laboratory has shown that ribosomes are attached to microtubules purified from unfertilized sea urchin eggs (Suprenant et al., 1989). The ribosomes are attached to the walls of the microtubules through a long tapered stalk of unknown composition. It is tempting to speculate that the stalk is entirely, or in part, composed of the EMAP. The stalks and ribosomes are removed in the presence of 0.35 M NaCl; this salt concentration also removes the 77,000 M_r MAP from taxol-stabilized microtubules (this report; and Vallee and Collins, 1986). Ribosomes are attached by a tapered stalk to egg microtubules purified from all four sea urchins used in this study (Suprenant et al., 1989). While it is only correlative, it is interesting that ribosomes are absent from microtubules isolated from surf clams, squid, bovine brain and mouse B16 cells; cells and tissues where the EMAP is also absent. These results indicate that the EMAP may be specifically involved in the interaction of the translational machinery with the cytoskeleton.

Messenger RNA is not randomly or uniformly distributed throughout the cytoplasm of eukaryotic cells. Recently, it was shown that the cytoplasmic localization of Vg1 RNA in *Xenopus* (Yisraeli et al., 1990) and bicoid RNA in *Drosophila* (Pokrywka and Stephenson, 1991) is dependent upon microtubule integrity. It is intriguing to speculate that we have purified a functional ribosome-microtubule complex from sea urchin eggs that is capable of binding and translocating specific mRNAs during early development.

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