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Characterization of a Novel Microtubule Cross-linking Protein from the Brine Shrimp, *Artemia*

by

Jianshe Zhang

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy
at
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International Law and Relations	0616
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Recreation	0814
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Medicine and Surgery	0564
Mental Health	0347
Nursing	0569
Nutrition	0570
Obstetrics and Gynecology	0380
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Ophthalmology	0381
Pathology	0571
Pharmacology	0419
Pharmacy	0572
Physical Therapy	0382
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Radiology	0574
Recreation	0575
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Toxicology	0383
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Polymer	0495
Radiation	0754
Mathematics	0405
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General	0605
Acoustics	0986
Astronomy and Astrophysics	0606
Atmospheric Science	0608
Atomic	0748
Electronics and Electricity	0607
Elementary Particles and High Energy	0798
Fluid and Plasma	0759
Molecular	0609
Nuclear	0610
Optics	0752
Radiation	0756
Solid State	0611
Statistics	0463

Applied Sciences

Applied Mechanics	0346
Computer Science	0984
Engineering	
General	0537
Aerospace	0538
Agricultural	0539
Automotive	0540
Biomedical	0541
Chemical	0542
Civil	0543
Electronics and Electrical	0544
Heat and Thermodynamics	0348
Hydraulic	0545
Industrial	0546
Marine	0547
Materials Science	0794
Mechanical	0548
Metallurgy	0743
Mining	0551
Nuclear	0552
Packaging	0549
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Sanitary and Municipal	0554
System Science	0790
Geotechnology	0428
Operations Research	0796
Plastics Technology	0795
Textile Technology	0994

PSYCHOLOGY

General	0621
Behavioral	0384
Clinical	0622
Developmental	0620
Experimental	0623
Industrial	0624
Personality	0625
Physiological	0989
Psychobiology	0349
Psychometrics	0632
Social	0451

TABLE OF CONTENTS

List of Figures

LIST OF TABLES

Abstract

A 49 kDa protein, previously proposed to cross-link microtubules (Campbell et al., 1989), was purified to apparent homogeneity from cell-free extracts of the brine shrimp, Artemia. When incubated with tubulin under assembly conditions, the purified 49 kDa protein cross-linked the resulting microtubules. The 49 kDa protein was moderately resistant to heat, and it did not react with antibodies to neural microtubule-associated proteins (MAPs) and kinesin. The 49 kDa protein cross-links microtubules in a nucleotide-dependent manner. Efficient removal of the 49 kDa protein from microtubules assembled in cell-free extracts of Artemia occurred with GTP and analogues of ATP, nonhydrolyzable or otherwise, but not with ATP itself. The latter nucleotide had a greater impact on cross-linking when microtubules were assembled from purified tubulin. The 49 kDa protein possessed a low level of nucleotidase activity, preferring either ATP or GTP as substrates. Unlike kinesin and dynein, the enzymatic activity of the 49 kDa protein was not stimulated by microtubules. Immunofluorescent staining of Artemia larvae by affinity purified antibodies localized the 49 kDa protein to mitotic spindles, midbodies and setal cells, all regions containing organized microtubules. Regions enriched in microfilaments were not stained with antibody to the 49 kDa protein , nor were microfilaments crosslinked by this protein in vitro. The 49 kDa protein, found in relatively constant amounts at all developmental stages examined, consisted of 4 - 5 isoforms with isoelectric points in the pH range 6.0 - 6.2. Isoform composition of the 49 kDa protein may be generated in part by phosphorylation, a posttranslational modification. The observations support the proposal that a novel family of proteins with the ability to modulate microtubule organization in a nucleotide dependent manner exists in Artemia.

LIST OF ABBREVIATIONS

AMP-PNP	5′-adenylylimidodiphophate
AMP-PCP	ß-γ-methyleneadenodenosine 5′-triphosphate
ATP	adenosine 5′-triphosphate
ATP-γ-s	adenosine 5′-o-(3-thiotriphosphate)
BCIP	5-bromo-4-chloro-3-indoxyl phosphate
BSA	bovine serum albumin
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
EDTA	ethylenediaminotetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)- -N,N,N,N,tetraacetic acid
FITC	Fluorescein isothiocyanate
GTP	guanosine 5′-triphosphate
HST	high salt tween
IEF	isoelectric focusing
kDa	kilodalton
LMW	low molecular weight markers
MAPs	microtubule-associated proteins
NBT	nitroblue tetrazolium
NP-40	nonidet-P40
P11	phosphocellulose
PBS	Phosphate buffered saline
Pipes	1,4-piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
rpm	rotations per minute
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TMED	N,N,N,N,-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)aminomethane
TRITC	Tetra rhodamine isothiocyanate
Tween-20	polyoxyethylene sorbitan monolaurate

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I. Introduction

1.1. Microtubule-associated proteins (MAPs)

Microtubules exist in eukaryotic cells as one of three principal filamentous elements of the cytoskeleton. They are long, hollow cylinders made of α- and β-tubulin arranged as a heterodimer with a molecular mass of about 100 kDa. A large number of heterogeneous proteins colocalize with cellular microtubules and they can be isolated with tubulin by temperature-dependent cycles of assembly and disassembly. These proteins are termed microtubule-associated proteins (MAPs) or microtubule-binding proteins (MBPs) (Olmsted 1986). The interaction of microtubules and MAPs is thought to influence cell properties such as shape, polarity, division, cytoplasmic organization, and intracellular vesicle transport (reviewed by MacRae, 1992 a,b; Wiche 1989; Matus 1990).

During the past two decades, the molecular characteristics and physiological functions of MAPs have been extensively investigated. Most of the early work was done on MAPs from mammalian brain and these are called the neural MAPs. These MAPs are usually divided into two groups: high molecular weight proteins, such as MAP-1 (300-350 kDa), MAP-2 (270 kDa), and MAP-3 (180 kDa) (Wiche, 1989), and the lower molecular weight tau proteins (50-60 kDa) (Lee, 1990). MAPs from tissues other than neural sources are designated as nonneural MAPs. The nonneural MAPs include many different

proteins, such as a family of mammalian MAPs each with a molecular mass of about 200 kDa and now termed MAP-4 (Chapin and Bulinski, 1992; Aizawa et al., 1990, 1989; Huber and Matus, 1990), a 67 kDa heat stable protein from bovine pancreas (Michalik et al., 1993), a 70 kDa MAP from starfish eggs (Hosoya et al., 1990), a 115 kDa (E-MAP-115) (Masson and Kreis, 1993) and a 170 kDa protein from HeLa cells (Rickard and Kreis, 1990). As the physiological functions of MAPs became known, they were divided into two functional classes, the architectural or structural, and the energy-transducing or mechanochemical MAPs (Campbell et al., 1989; MacRae, 1992b). The first group of MAPs either modulates microtubule polymerization, which is actively involved in the growth of cellular processes and the establishment of cellular morphology, or they form structures which crosslink microtubules to one another or to other organelles (Aamodt et al., 1989, 1986). The second class of MAPs includes the microtubule-based mechanochemical enzymes, such as kinesin and cytoplasmic dynein, and perhaps dynamin, a member of a new GTPase family of proteins (Nakada et al., 1993; Vallee and Sheptner, 1990). Some MAPs, such as MAP-1C and dynamin, appear to have both structural and energy-transducing functions (Shpetner and Vallee, 1989; Shpetner et al., 1988; McCaffery and Vale, 1989). Chapin and Bulinski (1992) proposed that all MAPs from higher organisms can be classified into two groups, based on whether their microtubule-binding is sensitive to

nucleotides. The authors further suggested that the nucleotide-sensitive MAPs, including kinesin, dynein and dynamin, have microtubule-activated ATPase or GTPase activity, while those proteins that are insensitive to nucleotides stimulate microtubule assembly, both in vivo and in vitro. This latter group of MAPs contains MAP1A, MAP1B, MAP2, tau, and MAP4, all of which are capable of influencing microtubule dynamics (Vallee 1990).

1.2. Structural MAPs

An important feature of the structural MAPs is their ability to promote tubulin polymerization. MAP-2 and tau from neural sources, and MAP-4 from nonneural tissues, stimulate tubulin assembly in vitro (see reviews by MacRae, 1992 a, b). Weingarten et al. (1975) observed that under assembly conditions in the absence of tau proteins, neural tubulin exists entirely as a 6S dimer of α- and β-tubulin. However, when tau proteins were added, tubulin polymerized into microtubules. Microinjection of tau into fibroblasts that have no endogenous tau proteins also greatly enhances tubulin polymerization (Drubin and Kirschner, 1986). MAP-2 directly alters the dynamic properties of tubulin assembly and disassembly by increasing the rate and length of the microtubule elongation phase and by reducing the rate and frequency of the shortening phase (Kowalski and Williams, 1993).

How do MAPs stimulate tubulin polymerization? Tau proteins are highly conserved with several isoforms translated from individual alternately spliced mRNA. They contain a C-terminal tubulin binding site with 3-4 repeats of 18 amino acids separated from one another by 13-14 amino acid residues (Himmler, 1989). Two of the three repeats, if synthesized in vitro and added to tubulin, cooperately promote microtubule assembly. The two assembly promoting regions are localized at tau (187-204) and tau (218-236) (Ennulat et al., 1989). MAP-2 has three isoforms, termed MAP-2a, MAP-2b (each about 270 kDa) and MAP-2c (70 kDa) (see review by Vallee, 1990) and these isoforms are tissue-specifically located and developmentally regulated (Kindler et al., 1990; Binder et al., 1984). MAP-2a and 2b can be digested into fragments of 30-40 kDa and 240 kDa. The smaller fragment binds to tubulin and promotes its polymerization, while the larger one forms arm-like projections that extend from the surface of microtubules (Harrison and Hyams, 1990). The assembly promoting fragment is derived from the C-terminus (Joly et al., 1989). Like tau, MAP-2 has three imperfect sequence repeats which constitute its microtubule-binding domain. Of the repeats, only the second one stimulates tubulin polymerization in vitro (Himmler et al., 1989; Lewis et al., 1989), while for tau, both the first and the second repeat sequences possess this ability The only difference between the first repeat sequence of tau and MAP-2, is that a lysine near the carboxyl terminus in MAP-

2 is substituted by a glycine in tau (Joly et al., 1989).

A heat-stable 190 kDa MAP (also termed MAP4) from bovine adrenal cortex promotes tubulin polymerization (Murofushi et al., 1986). A 27 kDa fragment obtained from the C-terminus of the 190 kDa by limited chymotrypic digestion consists mainly of three amino acids, lysine, proline and alanine and it contains repeated assembly-promoting and tubulin binding sites (Aizawa et al., 1987). The 190 kDa protein and tau contain at least one common sequence of 20 amino acid residues in their microtubule binding domains. A synthetic polypeptide based on these common sequences increased the rate of tubulin polymerization and decreased the critical concentration of tubulin required for polymerization (Aizawa et al.,1989). MAP2, tau and MAP4 share a similar physiological function, stimulating microtubule assembly via their microtubule-binding domains. Further as these domains are positively charged, while the C-termini of both α- and β-tubulin are acidic, it is likely that an ionic reaction occurs when these MAPs bind to microtubules and that the shielding of charges induces microtubule assembly (reviewed by Chapin and Bulinski, 1992; Paschal and Vallee, 1989).

Another feature of the structural MAPs is that they often form periodic projections which link microtubules to one another or to other cellular organelles, indicating a role for MAPs in maintaining structural organization of microtubule arrays in eukaryotic cells. The microtubule crosslinkers not

only organize microtubules into a functional network, but they also mediate microtubule-dependent cellular activities (reviewed by MacRae, 1992 a, b). Of the neuronal MAPs, cytoplasmic dynein (MAP-1C) (Amos, 1989), dynamin (Shpetner and Vallee, 1989), tau proteins (Hirokawa et al., 1988), MAP-2 (Lewis et al., 1989) and synapsin have microtubule crosslinking activity. MAP-1C and dynamin crosslink microtubules in a nucleotide-dependent manner and they show strong nucleotidase activities (Amos 1989; Shpetner and Vallee, 1989, 1992). When MAP-1C is incubated with microtubules, it forms phi-particles, which consist of two head-regions fused together with two separate tails, to connect microtubules into bundles (Amos, 1989). Dynamin is a 100 kDa protein initially purified from calf and rat brain and it crosslinks microtubules in vitro with a spacing between microtubules of about 13 nM (Shpetner and Vallee, 1989; Scaife and Margolis, 1990). By using quick-freeze, deep-etch and low angle rotary shadowing techniques, MAP-2 and tau were reported to interconnect microtubules into bundles and to form periodic arm-like structures, either projecting from microtubule surface or interconnecting adjacent microtubules. A recent report suggested that the flexibility of microtubules induced by taxol is reversed by both MAP-2 and tau, and that this effect may be caused by bridging microtubule protofilaments (Dye et al., 1993). Synapsin 1, a basic phosphoprotein, is capable of transporting synaptic vesicles to the presynaptic

terminus in neurons and it bundles microtubules and actin filaments by binding both tubulin and actin (Baines, 1989; Aubert-Foucher and Font, 1990). Whether these neural MAPs crosslink microtubules in vivo is uncertain.

There exists a large number of heterogeneous microtubule crosslinking proteins in nonneural cells (reviewed by MacRae, 1992 a). Two MAPs of 41,000 and 33,000 from Crithidia connect microtubules, but do not stimulate tubulin polymerization (Kambadur et al., 1990; Bramblett et al., 1989). The 41,000 kDa protein is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein shown to induce microtubule bundling (Huitorel and Pantaloni 1985; Kumagai and Sakai 1983). Trypanosoma brucei contains a 52-kDa protein which forms regular cross-linkers between microtubules in the presence of taxol and GTP (Ba aban et al. 1989). The 52 kDa protein may be a genuine cytoskeletal protein, but another trypanosome microtubule crosslinking protein of 41 kDa was identified as aldolase (Balaban and Goldman, 1990, 1989). Additionally, in sea urchin, MAPs bundle microtubules by forming microtubule cross-linkers (Hollenbeck et al., 1984).

MAPs from Crithidia form periodic crosslinkers along microtubules with a spacing of about 8.5 nm (Kambadur et al., 1990). In Caenorhabditis elegans crosslinkers occur at a frequency of one per tubulin dimer along protofilaments and the crosslinkers are usually about 5.7 nm long and 3.0 nm wide (Aamodt and Culotti, 1986). A 52 kDa protein from T.Brucei

forms crosslinkers with a centre to centre spacing of 7.2 nm when it coassembles with brain tubulin in the presence of taxol (Balaban et al.,1989). Sea urchin microtubule crosslinkers, on the other hand, have a periodicity along tubulin polymers of about 16 nm (Vallee and Bloom, 1983). Differences in crosslinker size and binding periodicity may reflect species variation and evolutionary modification, but they share the common properties of organizing microtubule networks.

1.3. Dynamic MAPs

Many motile activities in eukaryotic cells depend on the action of microtubule-based motor proteins. These mechanochemical enzymes couple the hydrolysis of nucleotides, such as ATP and GTP, to the generation of force for microtubule-related motility and organelle transport (Vale, 1990). Three proteins, kinesin, cytoplasmic dynein, and dynamin, were termed dynamic MAPs (MacRae, 1992 a, b), and they have been well characterized.

Kinesin and kinesin-like proteins

Kinesin is composed of two large polypeptides, each about 120 kDa, and two small peptides of 62 kDa. It consists of two small globular heads at one end, a "fan"-shaped tail at the opposite end and an elongated stalk in between (Kosik et al., 1990; Scholey et al., 1989). The two globular heads are

made up of heavy chains and they have a 400 amino acid domain at the N-terminal, which contains both the microtubule and ATP-binding sites (Gilbert and Johnson, 1993). The two light chains are localized in the tail, which may function as an attachment domain linking kinesin to membrane bound organelles or to chromosomes. The alpha helical coiled stalk region is formed by a portion of each heavy chain (McIntosh and Porter, 1989). Even though kinesin molecules vary in their structure, they retain their enzymatic activity in inducing microtubule movement *in vitro* (Yang *et al.*, 1990). Kinesin drives organelles toward the plus end of microtubules and it shows a microtubule stimulated ATPase activity ranging from 0.01-0.14 μmol/min/mg (Wagner *et al.*, 1989; Saxton *et al.*, 1988; Bloom *et al.*, 1988; Shimizu *et al.*, 1991). Besides MgATP, MgTTP, MgUTP, MgCTP and MgITP are hydrolysed by kinesin at a variety of rates and all nucleotides support kinesin-mediated microtubule sliding (Cohn *et al.*, 1989).

Kinesin is a highly conserved molecule (Yang *et al.*, 1989), but quite a large family of proteins share sequence similarity to the motor domain of kinesin and these are termed kinesin-like proteins (Klps) (Endow, 1991), or the kinesin superfamily. For example, at least 11 members of the kinesin superfamily were reported in *Drosophila* (Stewart *et al.*, 1991), In *Aspergillus*, the kat gene family includes at least five members and their sequences are similar to the ATP-binding and microtubule-binding motifs of kinesin (Mitsui *et*

al., 1993). In Xenopus, at least four Klps exist in the same cell and they are developmentally regulated (Vernos et al., 1993). These kinesin-like proteins have different molecular sizes and polypeptide compositions, participating in mitosis and meiosis, as well as other related cellular process, such as assembly of the mitotic spindle (Roof et al., 1992; Hyman et al., 1992; Nislow et al.,1992; Yen et al.,1992). For example, in Saccharomyces cerevisiae, two kinesin-related proteins, KIP1 and KIP2, mediate the migration of duplicated spindle pole bodies at the onset of mitosis and they are required for assembly of the mitotic spindles (Roof et al., 1992). Sawin et al.(1992) demonstrated that a kinesin-like protein, Eg5, from Xenopus eggs locates around the spindle poles. The extent of spindle formation was reduced by immunodepletion of Eg5, indicating a functional role for this protein in the mitotic process. A 312 KDa protein, CENP-E, functions in mammalian chromosome movement and /or spindle elongation. The protein attaches to kinetochores during chromosome congression, and the spindle midzone during spindle elongation (Yen et al., 1992). Other proteins, such as CBF3, CHO1, and KLP61F, belong to the kinesin superfamily and they are involved in mitosis and meiosis through binding to chromosomes and spindle microtubules (Nislow et al., 1992; Heck et al., 1993, Wright at al.,1993). These results suggest strongly that kinesin, or members of the kinesin superfamily, are responsible for spindle organization and chromosome

movement during mitosis and meiosis.

That kinesin cross-links microtubules as well as supporting dynamic events is suggested by molecular dissection of the molecule, wherein carboxyl- and amino-terminal domains of the protein bind microtubules independently of each other (Navone et al., 1992). Andrews et al. (1993) also reported that both the light chain and heavy chain ends of a kinesin molecule bind to microtubules to form a stable crossbridge. These observatic s reveal that kinesin functions in either crosslinking or sliding of microtubules, at least in vitro.

Cytoplasmic dynein

Dynein is the name given to another major group of microtubule-based energy transducing molecules, the first example of which was isolated from ciliary axonemal microtubules, and designated axonemal dynein (Gibbons, 1988). Axonemal dyneins consist of two or three heavy chains with molecular masses of 400-500 kDa, in addition to several intermediate chains (40-120 kDa), and some small chains (15-25 kDa) (McIntosh and Porter, 1989; Gibbons, 1988). Dyneins or dynein-like proteins isolated from microtubule preparations other than those from cilia and flagella are termed cytoplasmic dyneins. Cytoplasmic dyneins, with different molecular masses but similar biochemical and physiological properties, have been isolated from several organisms. Like flagellar dynein, cytoplasmic dynein contains two or three

heavy polypeptide chains (α, β, γ) and several small polypeptides and the heavy chains contain both microtubul binding and ATP-sensitive sites, which play an important role in microtubule-dependent motility activity (Zhang et al., 1993).

MAP-1C is a well-studied cytoplasmic dynein, which exhibits both microtubule crosslinking and microtubule stimulated ATPase activity. It is a 16-22S two headed protein complex composed of two large chains and several accessory polypeptides (Vallee and Shpetner, 1990; Vallee et al., 1988). MAP-1C is also an ATPase, but it hydrolyses CTP, TTP and GTP at higher rates than ATP (Shpetner et al., 1988). Cytoplasmic dynein from either rat tissues (Collins and Vallee,1989), sea urchin eggs (Grissom et al., 1992), or Dictyostelium exhibits CTPase activities as high as 444 nmol/min/mg (Koonce and McIntosh, 1990). Other dynein or dynein-like proteins occur in C. elegans (Lye et al., 1987), Reticulomyxa (Euteneuer et al., 1988), Paramecium (Schroeder et al., 1990), and squid (Gilbert and Sloboda, 1989). All of these proteins display similarities such as multiple polypeptide composition, a sediment coefficient of 14-20S, nucleotidase activity and microtubule-based motility.

Contrary to kinesin, cytoplasmic dynein moves membrane-bound organelles or vesicles in a retrograde fashion, that is, to the minus end of microtubules (Koonce and McIntosh,1990; Schnapp and Reese, 1989), although bidirectional transport

along microtubules was observed with cytoplasmic dynein from Paramecium and Reticulomyxa (Schliwa et al., 1991; Schroeder et al., 1990). Dynein requires activators to participate in microtubule-based cellular transport (Schroer and Sheetze 1991). Lacey and Haimo (1992) demonstrated that cytoplasmic dynein is a vesicle protein, directly bound to the vesicle membrane. The other cellular functions of cytoplasmic dynein involve assembly and transport of the Golgi apparatus (Theulaz et al., 1992), chromosome movement and nuclear segregation during mitosis (Eshel et al., 1993; Pfarr et al., 1990; Steuer et al., 1990) and lysosome movement (Lin and Collins, 1993, 1992). From the above description, it is clear that dynein plays a role in the spatial arrangement of cytoplasmic organelles (Vallee and Shpetner, 1990).

Dynamin

Besides its structural role as a microtubule cross-linker briefly described earlier, dynamin exhibits a high microtubule-stimulated GTPase activity (Shpetner and Vallee, 1990, 1989; Maeda et al., 1992). The GTPase is activated by acidic phospholipids and SH3 domains, which functions in mediating protein-protein interaction, similar to the way microtubules affect nucleotidase activity (Tuma et al., 1993; Gout at al., 1993). Recent studies revealed that dynamin is a phosphoprotein and it is phosphorylated by a protein kinase, which enhances its GTPase activity 12-fold (Robinson et al.,

1993). Dynamin is a single polypeptide of 100 kDa, possibly associated with a 70 kDa polypeptide cofactor (Scholey, 1990) and it can be removed from microtubules by both ATP and GTP. However, the combination of GTP and AMP-PNP is used to specifically isolate dynamin, because AMP-PNP causes kinesin to remain bound to microtubules in the presence of GTP. Dynamin prefers GTP to other nucleotides, such as ATP, CTP, UTP, and GDP as a substrate, and it shows a microtubule-stimulated GTPase activity as high as 1,979 nmol/min/mg (Hollenbeck, 1990).

The amino acid sequence of the gene products for rat brain dynamin shows no similarity to members of the kinesin superfamily or to neuronal MAPs (Obar et al., 1990), but it contains a domain of 300 amino acids that is similar to VPS1, responsible for yeast vacuolar protein sorting (Vater et al., 1992), to the Drosophila shibire gene product, which is associated with vesicle traffic (van der Bliek and Meyerowitz, 1991), to Vps1p, a protein associated with the Golgi apparatus (Wilsbach and Payne, 1993), and to a yeast SP015, functioning in meiosis (Yeh et al., 1991). Based on the sequence similarities just mentioned, dynamin is a member of a GTP-binding protein family (Hollenbeeck, 1990) with several possible physiological functions including synaptic vesicle transport (Vallee and Shpetner, 1993). Phosphorylation and dephosphorylation of dynamin may play a regulatory role in its function.

Chen et al.(1991) reported that dynamin exists in multiple tissue specific and developmentally regulated forms in Drosophila. Immunofluorescence staining with an antibody against rat brain dynamin revealed that in Drosophila, a 100 kDa dynamin-related protein occurs abundantly in heads, less abundantly in embryos, and is not present in adult bodies. Further experiments demonstrated that sea urchin 100-kDa dynamin-related protein is expressed throughout early embryonic development and then its synthesis decreases abruptly (Faire and Bonder, 1993). Nakata et al. (1993) reported that two dynamin-like proteins exist in the rat and they are expressed in a tissue-specific manner.

1.5. The interaction of MAPs with membrane-bound cellular organelles

Membranous organelles, such as mitochondria, the Golgi body, lysosomes, and endoplasmic reticulum, appear to associate with microtubules via MAPs. An important concern is to learn how these organelles interact with the cytoskeleton to effect a specific spatial organization.

Mitochondria move along microtubules, perhaps to position themselves in such a way so as to most effectively meet certain needs for cellular metabolism. In neurons, mitochondria move from the cell body into either the dendritic or axonal projection (Forman et al., 1987). In pinacocytes of the freshwater sponge, Spongillidae porifera, mitochondria

move along microtubules in almost straight paths, from the perinuclear region to the cell periphery, at an average speed of 1.3 μm/sec (Wachtmann et al., 1990; Weissenfels et al., 1990). Movement of mitochondria was also observed along insect ovarian microtubules (Dittman et al., 1987; Stebbings and Hunt, 1987). MAPs were reported to be involved in movement by forming crossbridges between mitochondria and microtubules (Rendon et al., 1990). Two major neuronal MAPs, MAP-2 and tau bind to purified rat brain mitochondria. MAP-2 was reported to bind mitochondria via its microtubule-binding domain and not by its projection domain (Jancsick et al., 1989). However, Linden et al.(1989) state that MAP-1 and MAP-2, but not tau, bind to mitochondria membranes through their arm-like projection domain . Mitochondria in the ovarian trophic cord of Dysdercus intermedius move along microtubules similar to nerve axonal transport (Dittman et al., 1987). Porin, a pore-forming protein may be the molecule which connects mitochondria to microtubules (Leterrier et al., 1990). These results demonstrate that both MAPs and microtubules directly or indirectly affect mitochondrion organization and function.

The Golgi body is actively involved in the intracellular transport, processing, and sorting of secretory and lysosomal proteins and the tight association of the Golgi with microtubules has been demonstrated in several systems (Kreis, 1990). The dynamics of microtubules influence the spatial distribution of the Golgi complex. Microtubules appear to act

as pathways for reorganization of scattered Golgi elements, since they move from the minus to the plus end of microtubules (Ho et al., 1989), and cytoplasmic dynein may be the motor involved in this ATP-powered translocation (Theulaz et al., 1992). So far two MAPs, a 110 kDa (Donaldson et al., 1990) and a 58 kDa protein (Bloom and Brashear, 1989) are known to link the Golgi apparatus to microtubules. The 110 kDa protein associates with the Golgi complex as a cytoplasmically oriented peripheral membrane protein and it mediates the interaction of the Golgi complex to microtubules. The protein is likely related to MAP-2, because it is recognized by a monoclonal antibody (M3A5) raised against MAP-2 (Allan and Kreis, 1986). The 58 kDa protein localizes to the cytoplasmic face of the Golgi complex and may serve as a site to anchor this organelle to microtubules. The protein cosediments with microtubules, stimulates tubulin polymerization and binds microtubules in a saturable manner. It is different from other MAPs in that it associates principally with the Golgi complex. By providing an anchorage site for microtubules on the outer surface of the Golgi complex, the 58 kDa protein leads to the intracellular positioning and structural integrity of this organelle (Bloom and Brasher, 1989).

Like mitochondria and the Golgi complex, lysosomes associate with microtubules. Reconstituted microtubules interact specifically with purified thyroid lysosomes and form two types of microtubule-lysosomes complexes in vitro: a

simple complex composed of a lysosome in close contact with a microtubule and a more complicated complex formed by a lysosome surrounded by several microtubules (Mithieux et al., 1988). A 50 kDa lysosome membrane protein from pig thyroid contains ATP and tubulin binding sites. This protein is thought to provide a stable but ATP-dependent attachment of lysosomes to microtubules Lin and Collins (1992) demonstrated that in cultured cells, cytoplasmic dynein and lysosomes are colocalized as detected by double antibody labelling and this association is disrupted by the treatment of cells with microtubule inhibitors. From the results, it seems that microtubules provide tracks for lysosome movement which is powered by dynein.

Other organelles, such as the endoplasmic reticulum (ER), nucleus and coated vesicles, depend, at least in part, on the microtubule network for their organization. Formation of the endoplasmic reticulum is directly or indirectly related to microtubule assembly and disassembly (Lee and Chen, 1988). Dabora and Sheetz (1988) observed that an ER-like tubulovesicular membrane network is formed in the presence of microtubules and ATP, indicating that microtubules and ER are interrelated organelles, and that movement of the ER is ATP-dependent (Kelly, 1990). During cell division, the nucleus usually migrates to the site of future division and microtubules appear to play an important role in migration (Katsuta et al., 1990). Cytoplasmic anchoring proteins are

reported to control nuclear localization and to influence movement. For example, a 22 kDa protein in Aspergillus nidulans acts as a crossbridge, linking the nucleus to microtubules, and it may be involved in the generation of force used to move the nucleus during interphase (Osmani et al., 1990; Hunt, 1989)

1.6, Artemia tubulin and MAPs

The brine shrimp, Artemia, is a crustacean of the class, Branchiopoda. It exhibits an unusual life history, in which the gastrula undergoes a relatively synchronous development without DNA synthesis and cell division. Mitosis resumes at emergence after about 12 h incubation at 27°C (Olson and Clegg, 1978) and mitosis resumes at about emergency stage (about 12 h of development at 27°C). Thus, Artemia has been the subject of studies on tubulin synthesis and microtubule organization during embryogenesis since developmentally-related changes in microtubules can be separated from cell-cycle related modifications (MacRae and Luduena, 1984). Tubulin from Artemia embryos was purified to apparent homogeneity by column chromatography and a cycle of assembly and disassembly (MacRae and Luduena, 1984), which provided a solid basis for subsequent study of microtubule proteins. Artemia contains 3 α- and 2 β-tubulin isoforms, as identified by Coomassie staining of two dimensional gels (Rafiee et al., 1986). The isotubulin composition, except for detyrosinated

tubulin, remains constant during postgastrula development (Langdon et al., 1991 a,b). In situ immunofluoresence staining with antibodies revealed that tubulin distribution may influence cellular polarity in Artemia (MacRae et al., 1991; Freeman at al., 1992).

Many non-tubulin proteins, or MAPs, coassemble with microtubules from Artemia (Rafiee et al., 1986). Microtubules, either induced by taxol to assemble in cell-free extracts of Artemia, or coassembled from unfractionated Artemia MAPs and purified tubulin, were crosslinked by MAPs (Campbell et al., 1989) and the authors suggested that a prominent protein with a molecular mass of 49 kDa is the most likely candidate for the microtubule crosslinker. The objective of my work was to determine if the 49 kDa protein is a microtubule crosslinker in Artemia and, if it is, to further characterize its properties by biochemical and immunological methods.

The 49 kDa protein has now been purified to apparent homogeneity from cell-free extracts of Artemia, allowing experiments which provided direct evidence of its microtubule crosslinking function, at least in vitro. Characteristics of the purified 49 kDa protein, such as its N-terminal amino acid sequence and heat stability, its ability to promote tubulin polymerization and microtubule crosslinking in vitro, as well as its relationship to other MAPs and kinesin, were determined. The 49 kDa protein crosslinks microtubules in a nucleotide dependent manner and exhibits a

limited nucleotidase activity not stimulated by microtubules. Immunolocalization of the 49 kDa protein *in situ* revealed that the protein occurs in mitotic spindles. The developmental profile of the 49 kDa protein during early *Artemia* development, its isoform composition and its presence in other species, were analyzed. The 49 kDa protein is a phosphoprotein and it is dephosphosphorylated by alkaline phosphatase. The results support the conclusion that the 49 kDa protein obtained from *Artemia* is a novel microtubule crosslinker and that it plays a nucleotide-sensitive role in microtubule organization.

II. Materials and Methods

2.1. Incubation of *Artemia* and Preparation of cell-free extracts

Fifty grams, dry weight, of dormant *Artemia* embryos (Great Salt Lake Brand) were hydrated in cold distilled water for at least five hours or overnight at 4°C. Hydrated cysts were collected on a Buchner funnel and washed with cold distilled water 2-3 times. For development, 20-30 g wet weight of embryos were suspended in 1-1.5 L of hatch medium (Appendix 1) and incubated at 27-28°C for 12-15 h with shaking at 220-250 rpm. Fifty-100 g amounts were homogenized with a Restsch motorized mortar with pestle (Brinkman Instruments Canada, Rexdale, Ont.). The homogenate was stirred for 20 min at 4°C, centrifuged at 16,000 g for 10 min at 4°C and the resulting supernatant, after passage through two layers of Microcloth, was centrifuged twice at 40,000 g for 30 min at 4°C in a JA-20 rotor, followed by two 30 min centrifugations at 210,000 g at 4°C in a Beckman 60 Ti rotor. The final supernatant, termed S1, was either used immediately or frozen at -70°C until needed.

To obtain organisms synchronized at instar I (17 h), instar II (33 h) and instar III (43 h), the animals were prepared as described by Langdon *et al.* (1990). Briefly, after 15 h of development, *Artemia* were poured into a round shallow dish, stirred to gather the organisms in the centre, and

placed in the dark with light shining from one side of the dish to attract the swimming nauplii. Those nauplii that moved to the light were drawn off after 10 min and discarded. After a further 50 min, nauplii attracted to the light were again drawn off and discarded. One h later, nauplii attracted to the light were harvested and those animals were synchronized at instar I. The first instar organisms were either homogenized immediately or were placed in a 1 L separatory funnel containing 800 ml of hatch medium. The mouth of the funnel was loosely covered and air was bubbled through the spigot of the flask. The Artemia were then grown for an additional 16 h or 26 h, yielding organisms at instar II and III of development, respectively. Following incubation, larvae were collected by filtration on 100 μm Nitex mesh (B.and S.H. Thompson Ltd.) and immediately homogenized by hand in 3-5 ml of Pipes buffer containing 4 M glycerol at pH 6.5. S1 was then prepared as described on the previous page for animals developed 15 h except that a cocktail of proteolytic enzyme inhibitors was added before homogenization. The inhibitors used were 0.2 mg of pepstatin A, 0.4 mg of phenylmethysulfonyl fluoride (PMSF), 0.4 mg of leupeptin and 0.4 mg of soybean trypsin inhibitor, all from Sigma Chemical Co., St Louis, MO.

2.2. Purification of the 49 kDa proteins

2.2.1. Preparation of Artemia cell free extracts (S1)

Preparation of Artemia (S1) for purification of the 49 kDa protein was as described in 2.1 except that all homogenization was done in a Retsch motorized mortar and pestle and each round of 49 kDa protein purification required three preparations of S1 from about 200 g of hydrated cysts.

2.2.2. Preparation of MAPs.

For the preparation of Artemia MAPs, S1 was thawed and centrifuged in a Beckman 60 Ti rotor at 210,000 g for 30 min at 4°C. Tubulin assembly was induced in S1 by addition of taxol to 10 μM and Mg-GTP to 1.8 mM followed by incubation at 37°C for 30 min. Taxol was the generous gift of Dr. Nancita R. Lomax, (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.). Eight to ten ml samples of S1 were loaded onto 20-25 ml, 15% sucrose cushions prepared in Pipes buffer (Campbell et al., 1989) containing 4 M glycerol and centrifuged at 40,000 g for 30 min at 28°C. Each of the pellets was rinsed gently twice with Pipes buffer at 37°C and resuspended in 250-300 μl of Pipes buffer containing 0.5 M NaCl. The microtubule suspension was incubated at room temperature for 35-45 min, followed by centrifugation at 40,000 g for 30 min at 20°C. The supernatant, desalted into Pipes buffer by passage through 5 ml Sephadex G-25 columns

(Campbell et al., 1989), was termed MAPs.

2.2.3. Heat treatment of MAPs.

The MAPs were heated for 5 min at 50°C, cooled on ice for 5-10 min, and centrifuged at 40,000 g for 30 min at 4°C. The cross-linking protein remained in the supernatant after heating and centrifugation, and the supernatant, termed heated MAPs, was either used immediately or stored at 70°C.

2.2.4. Phosphocellulose chromatography of Artemia MAPs.

Two preparations of heated MAPs were pooled (about 7 ml) and applied to a 5 ml Phosphocellulose P11 (Whatman, Mandel Scientific Co.Ltd, Lachine, Que.) column. The column was washed with Pipes buffer and the adhering proteins were sequentially eluted with Pipes buffer containing 0.15, 0.20 and 0.50 M NaCl. Proteins recovered with each buffer were desalted on Sephadex G-25 columns and analyzed in SDS-polyacrylamide gels. Their ability to cross-link microtubules was determined by examination of negatively stained samples from tubulin assembly mixtures with the electron microscope. The crosslinking activity was mostly present in the 0.2 M NaCl P11 fraction, which was used for further purification.

2.2.5. Ammonium sulfate fractionation

The 0.2 M NaCl fraction from Phosphocellulose P11 was brought to 50% $(NH_4)_2SO_4$ (2.8 g in 9.5 mL), stirred for 20 min

at 4°C, and centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was further fractionated by addition of $(NH_4)_2SO_4$ to 65% (3.8 g) and 90% (5.7 g). The pellets were resuspended in 0.5-0.7 mL of Pipes buffer, incubated on ice for 30 min, and desalted through Sephadex columns. The polypeptide composition and microtubule cross-linking activity of each fraction were determined by using SDS polyacrylamide gel electrophoresis and electron microscopy.

2.3. Preparation of Artemia Tubulin

Artemia tubulin was purified by ion-exchange chromatography using Phosphocellulose P11 and DEAE-cellulose (DE-52) (Whatman, Mandel Scientific) and cycling by assembly/disassembly (MacRae and Luduena, 1984). About 50 ml (1,500 mg of protein) of Artemia S1 was loaded on the Phosphocellulose P11 and the column was washed with Pipes buffer. The eluted proteins were pooled and applied to a DEAE cellulose column, previously washed with 1 M NaCl in Pipes buffer and equilibrated with Pipes buffer. The DEAE column was subsequently eluted with Pipes buffer, and with Pipes buffer containing 0.2 M and 0.3 M NaCl. The protein fractions eluted with 0.3 M NaCl were pooled, precipitated with 50% ammonium sulfate (29.1 g/100 ml) and centrifuged at 27,000 g at 4°C for 20 min. The precipitates were resuspended in 1 ml of Pipes buffer and dialysed overnight against Pipes buffer containing 8 M glycerol. Following the dialysis, the protein was diluted

1:1 with Pipes buffer and centrifuged at 40,000 g at 4°C for 30 min. The tubulin in the resulting supernatant was then collected by a cycle of assembly and disassembly in the presence of 1.8 mM GTP and 0.1 M $MgCl_2$ (MacRae and Luduena, 1984). The purified tubulin was stored at -70°C.

2.4. Preparation of Bovine Neural Tubulin

Bovine neural tubulin was prepared as described by MacRae and Luduena (1984). About 500-700 g of fresh brain tissue were homogenized in a Waring blender with Pipes buffer containing 4 M glycerol. The homogenate was subsequently centrifuged at 16,000 g and 40,000 g at 4°C for 10 and 30 min. Microtubule proteins in the supernatant were induced to assemble in the presence of 1.8 mM GTP at 37°C for 30 min. Microtubules, collected by centrifugation, were resuspended in Pipes buffer on ice for 30 - 40 min and centrifuged at 40,000 g at 4°C for 30 min. The tubulin in the supernatant was assembled again in the presence of 1.8 mM GTP for 30 min at 37°C. The microtubule pellets, resulting upon centrifugation at 40,000 g for 30 min, were resuspended in 1-5 ml of cold Pipes buffer and solubilized in ice for 30 min. The solubilized microtubule proteins after centrifugation for 30 min at 40,000g at 4°C were loaded on a 15 cm x 2.5 cm Phosphocellulose P-11 column previously washed with 1 M NaCl and equilibrated with Pipes buffer. The P-11 protein fractions, collected by washing the column with Pipes buffer, were dialysed overnight against

Pipes buffer containing 8 M glycerol at 4°C. The tubulin was then recovered by one cycle of assembly/disassembly, similar to the procedures for Artemia tubulin preparation.

2. 5. Heat stability of the 49 kDa protein

To determine its response to heat, a 50-65% $(NH_4)_2SO_4$ fraction of the crosslinking protein at 0.5 mg/ml in Pipes buffer was exposed to different temperature in a water bath for 5 min, immediately cooled on ice for 5-10 min and centrifuged at 40,000 g for 30 min at 4°C. The resulting supernatants were tested for their activity in microtubule-crosslinking, as determined by examination of negatively stained samples with the electron microscope. Supernatants and pellets were analyzed on 10% SDS polyacrylamide gels. The effect of bovine tubulin at 1.5 and 5.0 mg/ml, a combination of 10 mM dithithreitol (DTT) and 0.75 M NaCl, or a mixture of all three substances on the heat stability of the crosslinking protein was determined.

2.6. In vitro assays of microtubule crosslinking by coassembly of purified 49 kDa protein and tubulin

Purified 49 kDa protein at a concentration of 0.5-1.0 μg/μl was incubated at 37°C for 30 min in Pipes buffer with either Artemia or bovine tubulin at 1.0 mg/ml, GTP at 1.8 mM and taxol at 10 μM in assembly mixtures of 25-100 μl. After assembly, five μl samples were prepared for electron

microscopy, while the remainder of the mixture was centrifuged through a 500 µl, 15% sucrose cushion in Pipes buffer containing 4 M glycerol. The pellets were rinsed gently with warm Pipes buffer prior to preparation for electrophoresis. Occasionally, pellets were resuspended in Pipes buffer and examined by electron microscopy. The procedure, with minor modifications, was used routinely to assay microtubule crosslinking by the 49 kDa protein *in vitro* under different experimental conditions.

2.7. Peptide sequencing and synthesis

In preparation for amino-terminal sequencing, 50-60% $(NH_4)_2SO_4$ fractions containing the 49 kDa protein were electrophoresed on 10% SDS-polyacrylamide gels using the MZE 3328 IV buffer system of Moos *et al*. (1988) and then blotted to Immobilon membrane (Millipore) in CAPs buffer (Yuen *et al*., 1989). The 49 kDa bands, detected by staining with 0.1% Coomassie blue in 10% methanol, were cut from the blot. Sequencing was done directly from membrane in a Porton gas-phase microsequencer (model 2090) by the Biotechnology Service Center (Department of Clinical Biochemistry, The Hospital for Sick Children, Toronto, Ont.). Two samples were prepared and sequenced independently of one another. For comparison to known sequences three protein databanks including GenBank, the National Biomedical Research Foundation Protein Database, and the Swiss Protein Database were searched. The peptide

corresponding to the acquired sequence of 15 amino acids was synthesized by VetroGen Corporation (London, Ont).

2. 8. Nucleotide induced dissociation of microtubule cross-linking

The effects of nucleotides on cross-linking by the 49 kDa protein were determined for microtubules induced to assemble by the addition of 10 μM taxol to either cell-free extract of Artemia or to mixtures of purified tubulin and 49 kDa protein.

Microtubules, assembled in 860 μl samples of cell-free extract at 25 mg/ml from Artemia developed for 12 h, were collected by centrifugation at 40,000 g for 30 min at 20°C through 15% sucrose in Pipes buffer, rinsed gently with warm Pipes buffer, resuspended in 100 μl of 10 mM nucleotide solutions in Pipes buffer at room temperature for 30 min and then centrifuged at 40,000 g for 30 min at 20 °C. Pellets were rinsed and then resuspended in 100 μl of Pipes buffer, after which 50 μl from each of the suspensions, along with 50 μl of their corresponding supernatant, were resolved on 10% SDS polyacrylamide gels and blotted to nitrocellulose. The blots were immunostained by the alkaline phosphatase method using anti-49 kDa peptide antibody (described later).

Experiments with purified samples of Artemia tubulin and the 49 kDa protein were carried out as for the cell-free extracts although it was not necessary to blot the gels in

order to identify the 49 kDa protein. These experiments were also done by adding the nucleotide prior to incubation to see if microtubule cross-linking was inhibited. The reaction mixtures in a volume of 25 μl contained tubulin at 1 mg/ml, 49 kDa protein at 0.5 mg/ml and taxol at 10 μM. GTP, normally at a final concentration of 1.8 mM in reaction mixtures, was omitted.

The nucleotide-induced dissociation of microtubules and cross-linking protein was quantitated by scanning of gels and blots with an Abaton 300-GS scanner (Everex Systems, Inc., Fremont, CA). The resulting digitized images were analyzed with Scanning Analysis software (Biosoft, Ferguson, MO) on an Apple MacIntosh II computer. To determine the percentage of either cross-linker dissociation from or inhibition of binding to microtubules by a nucleotide the amount (in arbitrary units) of 49 kDa protein in the pellet and supernatant after exposure to that particular nucleotide were added. The percentage values were then calculated as the amount of 49 kDa protein in the supernatant as compared to the total amount (supernatant and pellet) in the reaction mixture. The results represent the average of three experiments.

2. 9. Nucleotidase assays

The hydrolysis of nucleotides by protein fractions generated during the purification of the 49 kDa protein was examined by use of a Malachite green based assay (Kodama et

al., 1986). Reactions were done in Pipes buffer at pH 6.5 and 37°C, conditions under which nucleotides disrupted microtubule cross-linking, with 49 kDa protein purification fractions at 0.5-0.8 mg/ml and nucleotide at 1-2 mM. Microtubules, when present, were assembled from purified Artemia tubulin at 37°C for 20 min, after which taxol was added to a final concentration of 10 μM and the incubation continued for an additional 10 min. This procedure produced longer, more normal appearing microtubules than were obtained when taxol was present from the beginning of tubulin assembly. The microtubules were collected by centrifugation at 40,000 g for 30 min at 20°C, resuspended in Pipes buffer and the protein concentration determined. To test their effect on nucleotide hydrolysis by the 49 kDa protein, microtubules were added to reaction mixtures to a final concentration of 0.3-0.5 mg/ml. When nucleotide hydrolysis rates were determined a reaction mixture of 150-250 μl was incubated at 37°C. Samples containing 1.2 μg of the 49 kDa protein fraction under study were withdrawn at timed intervals and analyzed for inorganic phosphate (Kodama et al., 1986). The hydrolysis rates, calculated from the linear portion of the resulting curves, are the average of 3 determinations.

2. 10. Immunological methods

2.10.1. Preparation of antibodies

Antibody to the 49 kDa protein was raised in both mice

and rabbits, with antigen preparation essentially as described by Kellog et al. (1989). In brief, $(NH_4)_2SO_4$ fractions containing the 49 kDa protein were electrophoresed in single-well, 10% SDS-polyacrylamide gels. Each gel was stained with Coomassie blue for 20 min, destained for 30 min, treated with 80 ml of 2% glutaraldehyde in H_2O for 1 h at room temperature with gentle agitation, and left overnight in 500 ml of 7% acetic acid to further destain. Gels were then washed twice with water for 30 min at room temperature, after which the 49 kDa protein band was excised from the gel, minced with a razor blade, mixed with 10 ml of water and homogenized in a motor-driven teflon dounce homogenizer. The homogenized gel was lyophilized and dissolved in 1-2 ml of sterile PBS, and either used immediately or frozen at -20°C until needed. For initial injections, the antigen was mixed with Freund's complete adjuvant, while subsequent inoculations at 2-week intervals were made with antigen mixed with incomplete adjuvant. Mice received four intraperitoneal injections before exsanguination, while rabbits were injected three to four times before bleeding.

Antibody was also produced by injecting rabbits subcutaneously with the amino terminal peptide of the 49-kDa protein after glutaradehyde-induced coupling of Keyhole Limpet haemocyanin (Sigma) as previously described (Gundersen et al., 1984; Bulinski et al., 1983). For initial injections antigen was mixed with Freund's complete adjuvant, while subsequent

injections at 2-week intervals were with antigen in incomplete adjuvant.

2.10.2. Affinity purification of anti-49 kDa protein antibodies

For affinity purification, antigen (partially purified 49 kDa protein) was resolved on 10% SDS polyacrylamide gels and then transferred to a nitrocellulose membrane. The band of 49 kDa protein were excised from the membrane and washed twice with TBS-tween buffer. The antigen containing membrane was incubated with either anti-49 kDa whole protein antiserum or anti-49 kDa peptide antiserum for 45-90 min at room temperature. After incubation, the membrane was rinsed two or three times with TBS-tween. Bound antibody was eluted with glycine buffer at pH 2.8 and immediately adjusted to pH 7.5 with phosphate buffered saline. Specificity was tested by reacting the purified antibodies with blots containing cell-free extracts and MAPs from Artemia.

2.10.3. Immunostaining of Western blots

After proteins were transferred to nitrocellulose (section 2.15), the membranes were incubated for 45-90 min with primary antibody diluted appropriately in TBS-tween, followed by washing the membranes three times (3-5 min). Secondary antibody, alkaline phosphatase conjugated (Sigma) and diluted with HST buffer (Appendix 1) to 1:2500-5,000, was

incubated with membranes at room temperature for 45-90 min. The blots were washed several times for 3-5 min with TBS-Tween, once with HST buffer and finally with TBS one time. Colour development was done by immersing the membrane in freshly prepared substrate solution (Appendix I) for an appropriate time. The reaction was terminated by placing the membrane in stop solution (Appendix 1) for 5-10 min, and it was then washed 2-3 times in water. Blots were photographed on a black background using Kodak Tri-X pan professional film.

2.10.4. Double immunofluoresecent staining of cross-linked microtubules

Crosslinked microtubules formed by coassembly of purified tubulin and 49 kDa protein were collected on coverslips by centrifugation (Mitchison and Kirschner, 1984). Following a brief rinse in PBS and fixation in cold methanol (-20°c) for 2 min, the coverslips were incubated for 45-90 min at room temperature with both DM1A, a general monoclonal antibody to α-tubulin, and an affinity purified antibody to the 49 kDa protein. The coverslips were then washed three times in PBS for 10 min and reacted with rabbit IgG-specific secondary antibodies conjugated with FITC at a dilution of 1:10 and a TRITC-conjugated antibody to mouse IgG diluted 1:20 in PBSAT. Secondary antibodies were from Sigma Chemical Co. The coverslips were washed three times in PBS for 10 min and mounted in 5% glycerol containing 0.1% amino-diphenylamine.

Slides were viewed in a Leitz Diaplux photomicroscope and photographed using Technical Pan 100 film.

2.10.5. Staining of cross-linked microtubules with gold-conjugated antibodies

Purified Artemia tubulin and the 49 kDa protein were coassembled as usual at 37°C for 30 min. Samples from each assembly mixture were placed on parafilm and grids were floated on them for 5 min. Following two washes of 5-10 min with Pipes buffer, the grids were fixed in 4% paraformaldehyde at room temperature for 5 min, and then rinsed twice with PBS containing 1% BSA. The grids were incubated with affinity purified antibody to the 49 kDa protein at room temperature for 45-90 min, rinsed twice with PBS containing 1% BSA, and then reacted with rabbit IgG-specific secondary antibody conjugated with 5 nm gold particles (Sigma Chemical Co.) diluted 1:100 at room temperature for 45-60 min. Following two rinses in PBS, the grids were refixed with 4% glutaraldehyde for 5 min and stained with 1% uranyl acetate in H_2O. The samples were viewed with the Philips 201 electron microscope.

2.10.6. Immuofluorescent staining of Artemia larvae

For immunofluorescent staining, Artemia larvae at instar I (17h), II (33h) and III (43h) were prepared as described in section 2.1. The larva were exposed to 2 μM taxol in hatch

medium for 15-20 min, followed by fixation for 4 h or overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at room temperature (MacRae et al., 1991). Following fixation, the larvae were rinsed twice with PBS, dissected, and placed in PBS containing 0.5% bovine serum albumin (BSA) and 0.5% Triton-X-100 (PBSAT). Primary antibody, diluted with PBSAT, was incubated with the larvae at room temperature for 1 h, and then overnight at 4°C. The anti-49 kDa peptide antibody was diluted 1:40, the antibody against 49 kDa whole protein was diluted 1:25, while DM1A was diluted 1:50 with PBSAT. After primary antibody incubation, the larvae were washed with PBSAT twice (30 min/each), and then incubated with FITC conjugated anti-rabbit IgG antibody and/or TRITC conjugated anti-mouse IgG antibody (Sigma Chem. Co.) at a final dilution of 1:40 for 4 h at room temperature. The specimens were washed twice with PBS for 30 min and mounted in 80% glycerol containing 0.1% amino-diphenylamine. For controls, the samples were exposed only to secondary antibodies. The specimens were viewed with a Leitz Dialux photomicroscope and photographed using Technical Pan 100 film.

2. 11. Fluorography

Fluorography was used mainly in the assay of protein phosphorylation and the identification of GTP-binding proteins. Briefly, for the assay of protein phosphorylation, P^{32}-labelled proteins were resolved in 10% SDS-polyacrylamide

gels. After electrophoresis, the gel was dried in an SGD 4050 Slab Gel Dryer at room temperature for 2-3 h, and then exposed to Kodak X-OMAT AR film with an intensifying screen at -70°C.

2.12. SDS polyacrylamide gel electrophoresis

One-dimensional sodium dodecylsulfate (SDS) polyacrylamide slab gels were used to analyze proteins according to the method of Laemmli (1970). Routinely, 10% separating gels were used at pH 8.8, while stacking gels were 4% at pH 6.8. Gels were run at a constant current of 30 mA/gel for 3-4 h and stained with either Coomassie blue or Silver (Sigma Chem. Inc.) following manufacturer's instructions (Technical bulletin no. P3040).

2.13. Isoelectric Focusing

Isoelectric focusing (IEF) was carried out essentially as described by Rafiee et al.(1986) and Langdon et al. (1990). IEF gels, composed of 4% acrylamide and containing 8 M urea were assembled in 12.5 cm glass tubes with internal diameters of 3 mm. A pH gradient of approximately 4.2-7.0 was established by using 0.5 ml of Bio-lyte 3/10 ampholyte (Bio-Rad). The gels were polymerized at room temperature for 1-2 h. Before electrophoresis, 20 μl of overlay buffer (Appendix 1) was applied to each gel and the gel was prefocused for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. The tops of the gels were washed with cathode solution (Appendix 1), and

protein samples were loaded with overlay buffer in a ratio of 1:1. Electrophoresis was carried out at 400 V for 14-16 h, followed by an additional one h at 800 V. Gels were stained with either Coomassie blue or silver. The pH profile obtained during isoelectric focusing was measured by sectioning of blank gels into 1 cm pieces which were placed in 1 ml of 0.025 M KCl for 2-3 h at room temperature before reading in a pH meter.

2.14. Two-dimensional gel electrophoresis

Isoelectric focusing gels were removed from glass tubes, denatured in treatment buffer (Appendix 1) for 2 h at room temperature, placed on the upper edge of 10% SDS polyacrylamide slab gels (11 cm x 16 cm x 1.5 cm) and embedded in 1% agarose in treatment buffer. Electrophoresis in the second dimension was at 30 mA/gel at 6°C for 3 - 4 h. The gels were stained with either Coomassie blue or silver.

2.15. Western blotting

Proteins were electrophoresed in 10% SDS polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell, BA85) according to the methods of Towbin *et al*. (1979). Transfer was carried out at 100 mA in blotting electrode buffer (Appendix 1) at room temperature overnight. After blotting, membranes were washed with TBS buffer (Appendix 1) once for 5 min, and then stained with 0.2%

Ponceau-S in 3% TCA to check transfer efficiency. The blots were then used for immunostaining with appropriate antibodies.

2. 16. Negative staining and TEM

Preparation of samples for electron microscopy was as described by Campbell at al.(1989). All samples were fixed in 4% glutaraldehyde in distilled water for 10-30 min and then placed on formvar-coated, carbon-stabilized grids. For negative staining, the grids were washed by floating upsidedown on a drop of Pipes buffer on parafilm, followed by successive transfer to two drops of distilled water and then to three drops of 1% uranyl acetate for 5-10 seconds each. Samples were then viewed in the Philips 201 electron microscope.

2.17. Protein phosphorylation and dephosphorylation

The procedures for the phosphorylation of the 49 kDa protein were as described by Correas et al.(1992) with minor changes. The 49 kDa protein, at 0.3-0.5 $\mu g/\mu l$, was incubated with cAMP-dependent kinase (Sigma) (0.1-0.5 $\mu g/\mu l$) at 37°C for 30 min in 100 mM Pipes, 0.5 mM $MgCl_2$, 2 mM EGTA, 10 mM cAMP, 1 mM PMSF and [γ-^{32}P]ATP at a final concentration of 0.5-1.0 mM. After incubation, the reaction mixtures were boiled immediately with treatment buffer and resolved in 10% SDS polyacrylamide gels. The gels were dried in a SGD 4050 Slab Gel Dryer for 2-4 h and exposed to Kodak X-OMAT AR X-ray film.

Dephosphorylation of the 49 kDa protein was performed as described by Hisanaga et al. (1993). Samples of purified 49 kDa protein (0.3-0.5 $\mu g/\mu l$) were dephosphorylated in 20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM EDTA by alkaline phosphatase (Sigma Chemical Co.) (100 μ/ml) at 37°C for the appropriate times. For acid phosphatase, enzymatic reactions were carried out in 10 mM Pipes (pH 6.0), 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM EDTA. All dephosphorylations, with either alkaline or acid phosphatase, were carried out in the presence of proteolytic inhibitors including 20 and 25 μl of inhibitor solution A and B (Appendix) respectively per ml of reaction mixture. After incubation, samples were boiled, electrophoresed in 10% SDS polyacrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose and stained with antibody. In microtubule binding assays, dephosphorylated 49 kDa protein, upon treatment with the phosphatase inhibitor, okadaic acid, at concentrations from 1-3 mM, was coassembled with either purified bovine or Artemia tubulin at 37°C for 30 min in the presence of 10 μM taxol and 1.8 mM GTP. Binding of the dephosphorylated 49 kDa protein to microtubules was determined by centrifugation, followed by electrophoresis of the resulting pellets and supernatants on 10% SDS polyacrylamide gels. The SDS gels were either stained with Coomassie blue or they were blotted to nitrocellulose and immuostained with anti-49 kDa peptide antibody. Microtubules coassembled from

the dephosphorylated 49 kDa protein and tubulin were viewed in the electron microscope.

2. 18. Analysis of various species for the 49 kDa protein

To determine if proteins immunologically related to the 49 kDa protein occur in other species, cell-free extracts were prepared from diatoms, snails, fruit flies, fish (Tilapia), chicken and cows. Cultures of the diatom, Thalassiosira fluriatilis, were kindly provided by N. Machell (summer student) in our laboratory. Cell-free extracts from both snail foot and brain were prepared by Andrew Jackson, a summer student. The homogenates from brain were applied directly to the gels, while those from feet were centrifuged before use. Fruit flies (Drosophila melanogaster), were cultured in yeast-supporting medium at room temperature. Organisms at the pupa stage were collected and homogenized in Pipes buffer containing 4 M glycerol. The brains and testis from fish, (Tilapia), were homogenized in a motor and pestle for 8-10 min in the presence of proteolytic enzyme inhibitors. Chicken embryos, developed for 15 days at 37°C, were demembraned, rinsed in physiological saline at pH 7.0 and homogenized in a motor and pestle. Bovine brain and Artemia were homogenized as previously described. These organisms were chosen readily available. Some of them were in developmental stages and thus similar to Artemia. Others, such as nerual tissues, are known to have large number of crosslinking microtubules.

All homogenates, unless otherwise noted, were centrifuged twice in a JA20 rotor at 40,000 g for 30 min at 4°C. Microtubule proteins in each preparation were induced to assemble in the presence of 10 μM taxol and 1.8 mM GTP at 37°C for 30 min. The assembled microtubules, collected by centrifugation, were assayed by SDS polyacrylamide electrophoresis and/or Western blotting. Samples were also removed after assembly, fixed with glutaraldehyde, negatively stained and examined in the electron microscope.

2. 19. Proteolytic digestion of the 49 kDa protein

The purified 49 kDa protein (0.5 μg/μl) was incubated with papain (Sigma Chemical Co.) at concentrations from 0.3-1.0 μg/ml in Pipes buffer at 37°C for appropriate times. Digested samples were analyzed by electrophoresis in 10% SDS polyacrylamide gels.

III, Experimental Results

3. 1. Purification of the 49 kDa protein

To isolate of the 49 kDa protein, microtubules were induced to assemble by addition of 10 μM taxol and 1.8 mM GTP to Artemia S1, and collected by centrifugation through 15% sucrose cushions in Pipes buffer (Fig.1). MAPs were then eluted from the pelleted microtubules with 0.50 M NaCl in Pipes buffer (Vallee, 1982; Campbell et al., 1989). A survey was carried out to determine the optimum conditions for extraction and the results showed that MAPs could be best separated from microtubules by incubation with 0.5 M NaCl in Pipes buffer at pH 6.5 for 40-45 min. When the MAPs were heated at 50°C for 5 min, followed by cooling on ice, a large number of polypeptides were removed upon centrifugation at 4°C at 40,000 g for 30 min, while the 49 kDa protein remained in the supernatant (Fig.1). A survey of the heat stability of the 49 kDa protein revealed that the protein resisted temperatures as high as 65°C (see section 3.3 and Fig.4 for results of these experiments). The heated MAPs were loaded on a Phosphocellulose P11 column which was washed with a series of buffers of increasing NaCl concentrations. The 49 kDa protein eluted in the narrow range of 0.15-0.2 M NaCl in

Figure 1. Purification of the Artemia 49-kDa protein. (A) Coomassie blue stained gel of protein fractions obtained during the purification of the 49 kDa protein. Lane 1, 106 μg of Artemia S_1; lane 2, 64 μg of Artemia MAPs; lane 3, 50 μg of heated Artemia MAPs after centrifugation; lane 4, 14.8 μg of the 0.2 M NaCl P11 fraction; lane 5, 9.8 μg of the 50-65% $(NH_4)_2SO_4$ fraction; lane 6, molecular weight markers from top to bottom 97,400, 66,200, 42,700, 31,000, 21,500 and 14,400. (B) Silver-stained gel of protein fractions obtained during the purification of the 49-kDa protein. (C) Western blot of protein fractions obtained during purification of the 49 kDa protein and stained with the antibody raised to the 49 kDa protein amino terminal peptide. Equivalent fractions in each panel have the same number and the amounts of protein in corresponding fractions were about equal to one another. The arrowhead in A indicates a protein that sometimes contaminated the $(NH_4)SO_4$ fraction, while in C it indicates the location of a polypeptide that cross-reacted weakly with an antibody to the 49 kDa protein.

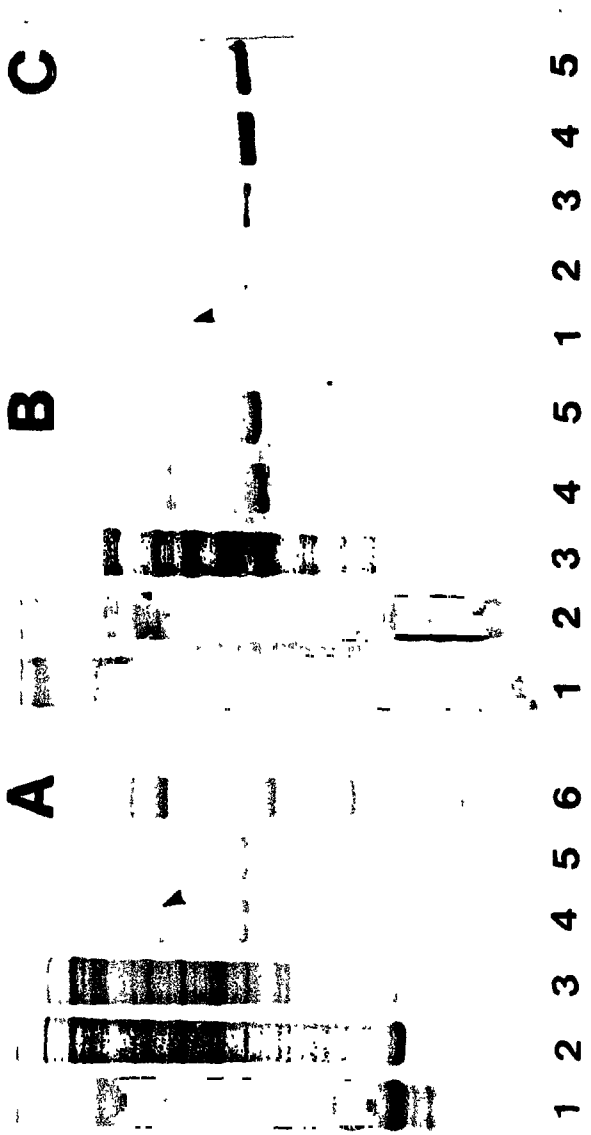

Figure 1.

Pipes buffer (termed the P11 fraction) (Fig.1, lane 4). The P11 fraction was further subdivided by addition of ammonium sulfate with the 49 kDa protein precipitating in the 50-65% $(NH_4)_2SO_4$ fraction (Fig.1, lane 5). Protein recovery presented as the average of three experiments, is given in Table 1.

From both Coomassie and silver stained gels (Fig.1, A and B), the 49 kDa protein was always a major component of the 0.2 M NaCl fraction from Phosphocellulose and was usually the sole protein visible in the 50-65% $(NH_4)_2SO_4$ fraction (Fig.1, lane 5). Occasionally a second protein (indicated by the arrowhead in lane 4 of fig.1A) was present in the 50-65% $(NH_4)_2SO_4$ fraction, but microtubule crosslinking in its absence indicated that it was not required for this reaction. An antibody raised in rabbit to a peptide corresponding to the amino terminal 15 amino acids of the 49 kDa protein (described later in the thesis) reacted with a similar sized protein on Western blots of all protein fractions except for S1 (Fig.1C). The antibody recognized only one polypeptide other than the 49 kDa protein, and this so weakly that it did not survive photographic reproduction (arrowhead, lane 1 of Fig.1C). A second antibody, raised in mouse to the entire protein, also failed to react with the 49 kDa protein in S1, although it recognized this protein in other purification fractions (data not shown). The mouse antibody did not interact with the weakly stained polypeptide detected by the anti-peptide antibody. The absence of a reaction with the

Table 1. Protein recovery during purification of the 49 kDa protein

Fraction	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Protein recovery (%)
S1	76.0±8.0	26.5±4.0	2014.0±110	100
MAPs	8.0±1.0	3.2±1.0	25.6±3.0	1.30
Heated MAPs	7.4±1.0	2.5±0.2	18.5±0.5	0.90
0.2 M P11	9.5±2.0	0.2±o.1	1.9±0.1	0.09
50-65% $(NH_4)_2SO_4$	0.5±0.1	0.5±0.1	0.3±0.1	0.01

The table represents the yield from 300 g (wet weight) of Artemia developed for 12 h. The values reported are the average of three experiments and they include the standard deviations. Preparation of fractions is described in Materials and Methods.

the 49 kDa protein in S1 was taken as an indication that very small amounts of this protein occur in cell-free extracts.

3.2. Demonstration that the 49 kDa protein is the microtubule crosslinker.

When the 50-65% $(NH_4)_2SO_4$ fraction was coassembled with either Artemia or bovine neural tubulin, the 49 kDa protein cosedimented with microtubules upon centrifugation through 15% sucrose cushions (Fig.2). Microtubules from assembly reactions containing the 49 kDa protein, even after centrifugation through sucrose, were clearly crosslinked (Fig.3) and tended to aggregate into large clumps, while microtubules assembled without the 49 kDa protein displayed neither of these behaviours. The particles connecting crosslinked microtubules were of differing morphologies (Fig.3). A bilobed particle is seen in Fig.3a (arrowhead), while in Figs. 3b and 3c, single round particles are indicated by the arrowheads. The remaining particles were elongated and were either numerous (Figs. 3a and 3b) or more sparsely disposed (Fig. 3c) between the microtubules. The crosslinker tended to join microtubules in pairs, even when small clumps of microtubules were observed (Fig. 3d). The exception to the pairing of microtubules in Fig.3d occurred in a region where microtubules were damaged.

Figure 2. Cross-linked microtubules are composed of tubulin and the 49 kDa protein. Artemia and bovine brain tubulin were assembled in the presence of the 50-65% $(NH_4)_2SO_4$ fraction and 10 μM taxol, after which the microtubules were centrifuged through 15% sucrose cushions. The pellets and supernatants were resolved on 10% SDS-polyacrylamide gels and then stained. (A) Coomassie blue stained gel. Lane 1, purified Artemia tubulin; lane 2, purified bovine tubulin; lane 3, 12 μg of the purified 50-65% $(NH_4)_2SO_4$ fraction; lanes 4 and 5, pellets from incubations of the 49 kDa protein with Artemia and bovine brain tubulin, respectively; lanes 6 and 7, supernatants from incubations of the 49 kDa protein with Artemia and bovine brain tubulin, respectively; lane 8, molecular weight markers as described in Fig. 1. (B) Silver staining of the gel shown in A subsequent to Coomassie staining.

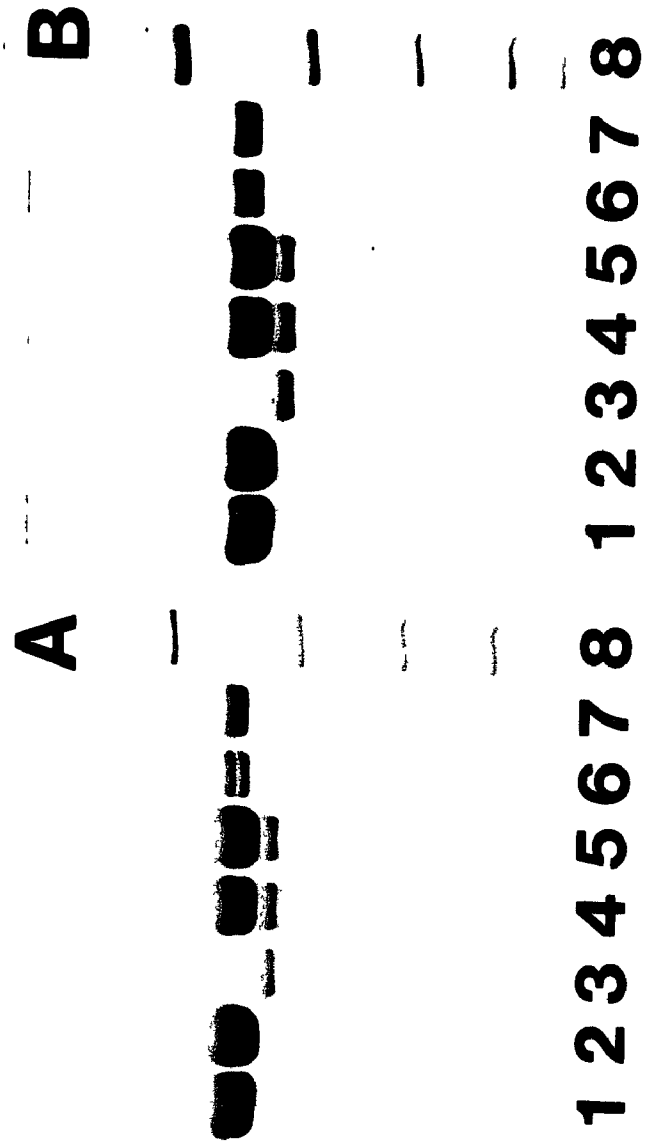

Figure 2.

Figure 3. Morphology and distribution of particles formed by the microtubule cross-linking protein from Artemia. Artemia tubulin was assembled in the presence of the purified 49 kDa protein and the microtubules, centrifuged through sucrose, were fixed with glutaraldehyde, negatively stained with uranyl acetate, and examined by electron microscopy. The smaller arrowheads identify particles of specified morphologies, as described in Results. The large arrowheads in d indicate a region where microtubule structure is disrupted, while the dashed white lines delineate microtubule pairs. The bar in A represents 0.1 μm and all magnifications are the same.

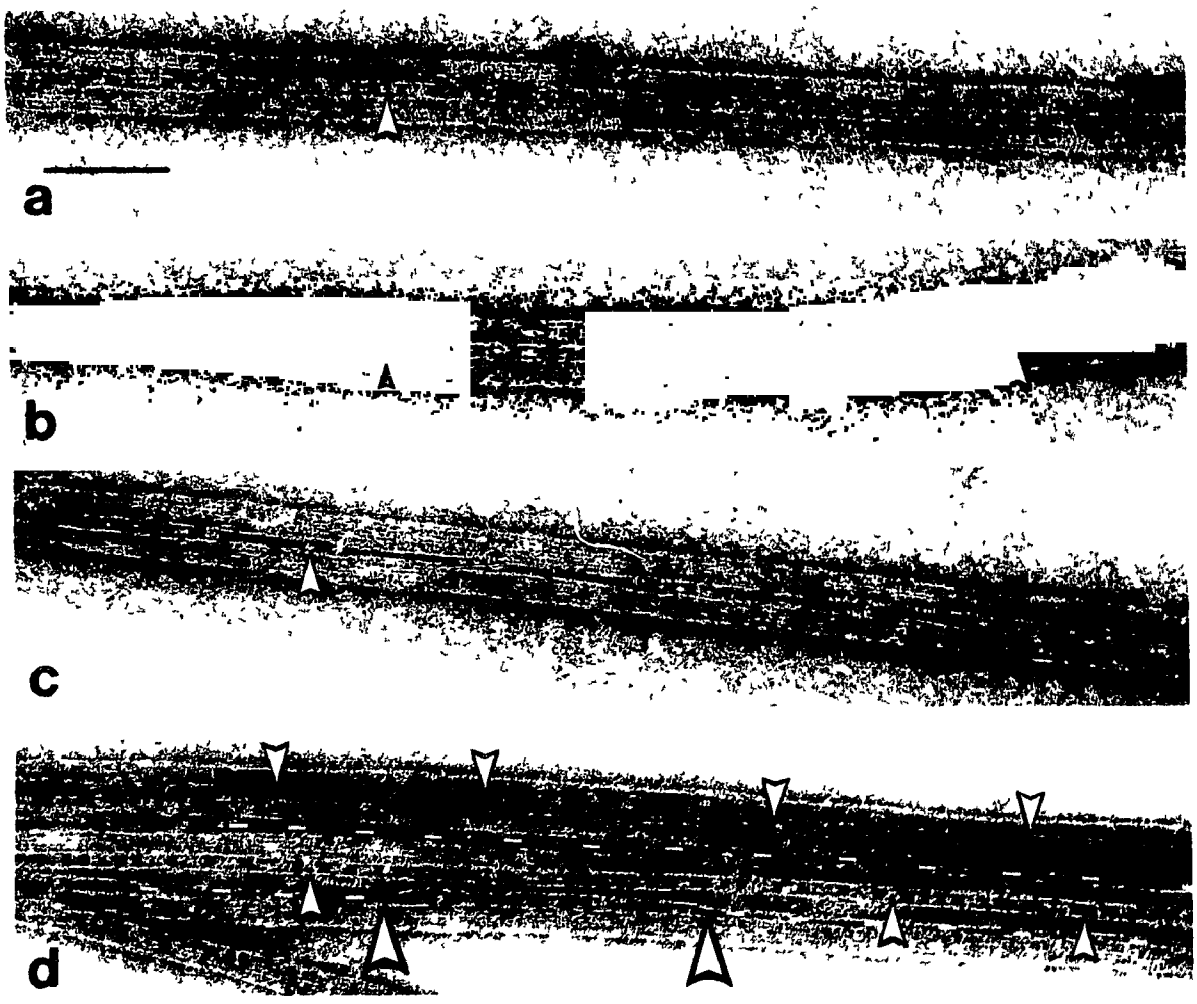

Figure 3.

3. 3. Heat stability of the 49 kDa protein

When heated in Pipes buffer for 5 min the crosslinking protein began to precipitate at 55-60°C, a process which was almost complete at 70°C, with only a very small amount of the 49 kDa protein left in solution after heating at this temperature (Fig.4A). Correspondingly, qualitative assessment of negatively stained samples with the electron microscope, showed a decrease in crosslinking as the temperature was increased. When the crosslinking protein was heated at 55-60°C, it coassembled with tubulin (Fig. 4B) and induced crosslinking, but after heating over 60°C these properties of the 49 kDa protein were lost. The supernatants obtained after heating at 65°C contained a substantial amount of cross-linking protein, indicating loss of function did not necessarily entail precipitation from solution. Addition of 10 mM DTT and 0.75 M NaCl afforded slight protection for the cross-linking protein against heat induced precipitation, even at 100°C for 5 min (not shown). Addition of tubulin at either 0.5 or 1.5 mg/ml caused complete precipitation of the 49 kDa protein, either in the presence or absence of DTT and NaCl, when the mixtures were placed in a boiling water bath for 5 min and then immediately cooled.

Figure 4. Heat stability of the 49 kDa microtubule cross-linking protein from Artemia. A. Artemia MAPs fractions, containing 20 μg of protein, were heated for 5 min, immediately cooled and centrifuged. Resulting pellets (lanes 2-6) and their corresponding supernatants (lanes 7-11) were electrophoresed on SDS polyacrylamide gels and stained with Coomassie blue. The temperatures employed were 50°C, lanes 2 and 7; 55°C, lanes 3 and 8; 60°C, lanes 4 and 9; 65°C, lanes 5 and 10; 70°C, lanes 6 and 11. Lane 1 shows MAPs used in this experiment before they were heat treated. The arrowhead indicates the location of the 49 kDa cross--linking protein. B. Artemia (lane 1) and neural (lane 2) tubulin were coassembled with the supernatant from MAPs heated to 55°C. The pellets were electrophoresed on SDS polyacrylamide gels and stained with Coomassie blue. The arrowheads indicate the 49 kDa cross-linking protein. tub, tubulin.

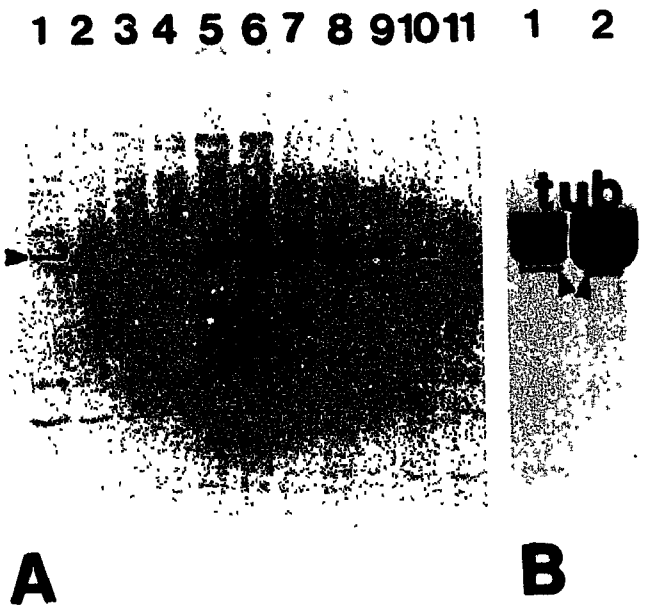

Figure 4.

3.4. Amino-terminal sequence of the 49 kDa protein

The first 15 amino-terminal amino acid residues of the 49 kDa protein were determined to be Phe-Tyr-Ser-Tyr-Ser-Gln-Glu-Pro-Phe-His-Pro-Ile-Gln-Gly-Arg. A computer search of three databanks, named in Materials and Methods, failed to identify another protein with a similar amino terminus. As revealed by electron microscopy and gel electrophoresis, a five-fold molar excess of the amino-terminal peptide had no effect on microtubule crosslinking in assembly reactions containing tubulin and the 49 kDa protein (data not shown).

3. 5. Immunological comparison of the 49 kDa protein to neural MAPs and kinesin

Polyclonal antibodies raised in mice and rabbits to either the 49 kDa protein or to its amino terminus failed to recognize neural MAPs and kinesin under conditions where they reacted very well with crosslinking protein (Fig.5).
These results, in concert with the observation that antibodies to tau, MAP2 and kinesin did not recognize the 49 kDa protein, even through they reacted with their respective antigens on companion Western blots, indicated that the crosslinking protein is unrelated immunologically to the major neural MAPs and to kinesin.

Figure 5. Immunological comparison of the 49 kDa protein to bovine neural MAPs and to kinesin. Proteins were electrophoresed on 10% SDS-polyacrylamide gels, blotted to nitrocellulose, and immunostained by the alkaline phosphatase method. Upper panel: lane 1, bovine neural microtubule proteins after three cycles of assembly and disassembly; lane 2, Artemia MAPs. (A) Control blot exposed to anti-mouse IgG secondary antibody, but not to primary antibody (the same result was obtained with the anti-rabbit IgG secondary antibody); (B) anti-MAP2 (4F7); (C) anti-MAP2 (5F9); (D) anti-tau (5E2); (E) anti-49 kDa protein antibody raised in rabbit to the amino-terminal peptide of the 49 kDa protein; (F) anti-49 kDa protein antibody raised in rabbit to the entire 49 kDa protein. Lower panel: lane 1, Artemia S1; lane 2, Artemia MAPs; lane 3, the 49 kDa protein; lane 4, kinesin. (A) anti-49 kDa protein antibody raised in mouse to the entire 49 KDa protein; (B) anti-kinesin; (C) control blot exposed to anti-mouse IgG secondary antibody, but not to primary antibody. With the exception of the arrowhead in B of the lower panel, which indicates kinesin, all arrowheads point to the 49 kDa protein.

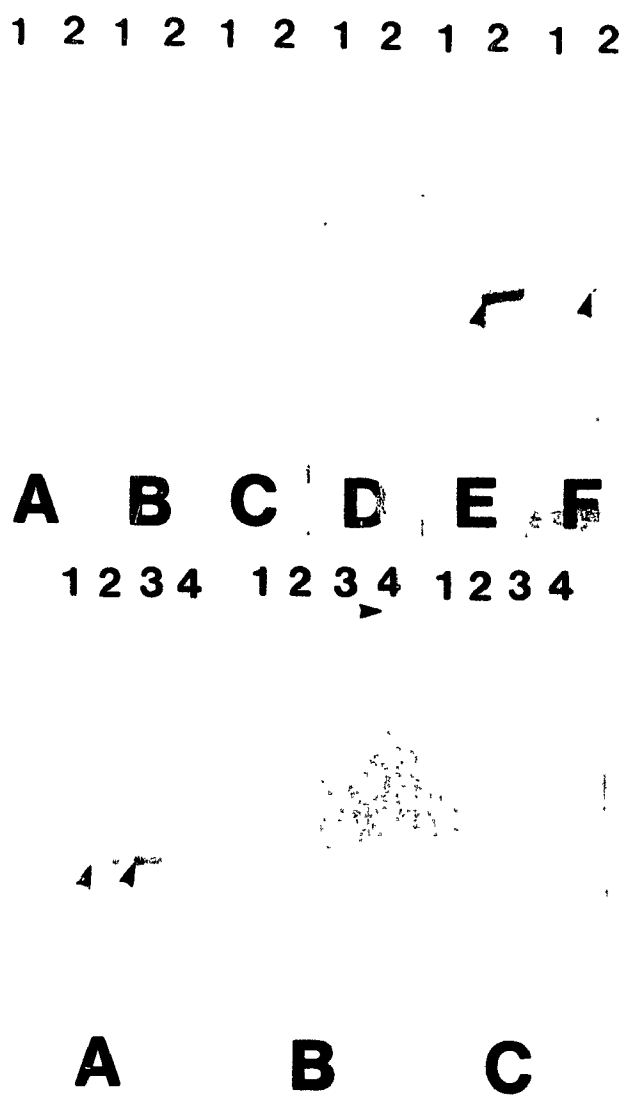

Figure 5.

3. 6. Nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled in cell-free extracts of Artemia

Microtubules assembled in cell-free preparations of Artemia developed for 12 h were collected by centrifugation through sucrose cushions, extracted with nucleotide-containing or control solutions and then recentrifuged to yield pellet (microtubules) and supernatant (MAPs) fractions. These samples were electrophoresed on SDS polyacrylamide gels and either Coomassie stained (Fig.6, upper panel) or blotted to nitrocellulose and immunostained with an antibody raised to the 49 kDa protein (Fig. 6. lower panel). Fig.6 shows results from a typical extraction with some of the nucleotides tested. Experiments with these, and additional nucleotides, were done in triplicate and are presented quantitatively in Fig.7. Microtubule pellets, even after extraction with Pipes buffer containing 0.5 M NaCl consisted of many proteins in addition to tubulin (Fig. 6, upper panel). However, essentially all of the 49 kDa protein was extracted with 0.5 M NaCl, whereas almost none was removed from microtubules with Pipes buffer lacking NaCl (Figs. 6,7). Of the nucleotides examined for dissociation of the 49 kDa protein from microtubules assembled in cell-free preparations, GTP was the most effective, followed closely by AMP-PNP and ATP-γ-s, then GDP and UTP. The remaining nucleotides, including ATP and one of its analogues (ATP-PCP), removed about 20% or less of the 49 kDa

Figure 6. Nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled in cell-free extracts of Artemia. Microtubules, induced to assemble in cell-free extracts of Artemia, were collected by centrifugation through sucrose cushions, resuspended in 10 mM nucleotide solutions at room temperature for 30 min and centrifuged. Pellets (P) and supernatants (S) were resolved on SDS polyacrylamide gels and Coomassie stained (upper panel) or blotted to nitrocellulose and immunostained by the alkaline phosphatase method (lower panel). Corresponding pellets and supernatants share the same number and were the result of extraction by 1, Pipes buffer; 2, 0.5 M NaCl; 3, ATP; 4, GTP; 5, CTP; 6, AMP-PNP; 7, ATP-r-S. T, tubulin; C, 49 kDa microtubule crosslinking protein. The size (X 10^{-3}) of the molecular weight markers is indicated on the right side of the gel.

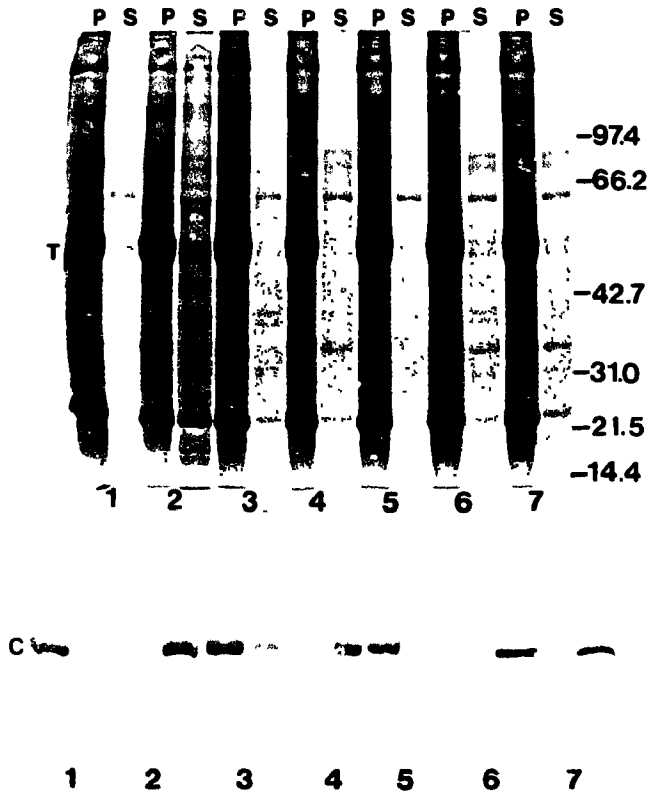

Figure 6.

Figure 7. Quantitation of nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled in cell-free extracts of Artemia. Blots prepared as described in Fig. 6 were scanned with the Abaton 300-GS scanner providing a measure of 49 kDa protein in corresponding supernatants and pellets after each treatment. The percent extraction reported is the amount of 49 kDa protein released into the supernatants as compared to the total quantity of 49 kDa protein in the pellet and supernatant. Data are the mean ±S.D. of 3 experiments. The extractions were done with: 1, Pipes buffer; 2, ATP; 3, ADP; 4, AMP; 5, GTP; 6, GDP; 7, UTP; 8, CTP; 9, AMP-PNP; 10, AMP-γ-s; 11, AMP-PCP; 12, cAMP; 13, 0.5 M NaCl.

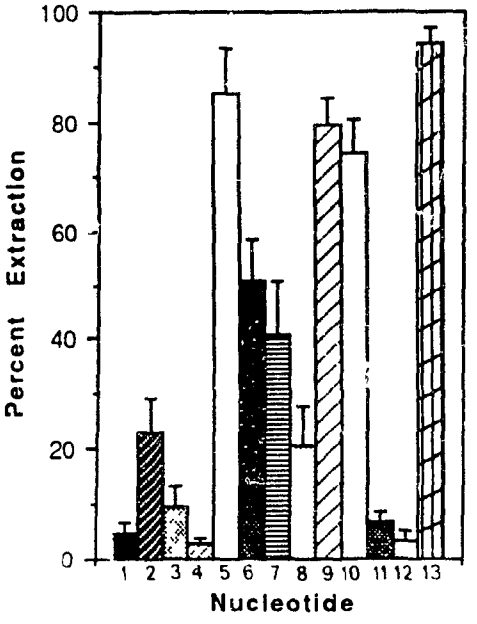
100
80
60
40
20
0
Percent Extraction
1 2 3 4 5 6 7 8 9 10 11 12 13
Nucleotide

Figure 7.

Figure 8. Electron micrographs of microtubules assembled in cell-free extracts of Artemia after exposure to nucleotide solutions. Microtubules were induced to assemble in cell-free extracts by addition of taxol, collected by centrifugation through sucrose, treated with nucleotides, recentrifuged, and prepared for electron microscopy after resuspension in buffer. Panel A, microtubules before extraction; B and C, microtubules after extraction with ATP and GTP respectively. Arrowheads, cross-linking particles; arrows, structurally abnormal microtubules; lower case a, amorphous material. The bar in A represents 0.2 μm and the magnification in all figures is the same.

Figure 8.

protein. Decreasing the number of phosphates in adenine-and guanine-containing nucleotides reduced their ability to dissociate the 49 kDa protein from microtubules.

Prior to nucleotide treatment microtubules assembled in cell-free extracts of Artemia were associated with densely staining, amorphous material probably composed of precipitated proteins and they were connected to one another by particles visible with the electron microscope (Fig. 8A). After exposure to ATP or to other nucleotides which extracted the 49 kDa protein inefficiently, it was still relatively easy to find crosslinked microtubules (Fig. 8B). On the other hand, nucleotides that effectively removed the 49 kDa protein from microtubules left very few of the polymers crosslinked (Fig. 8C). The amorphous material was mostly removed (compare Fig. 8A to Fig. 8B and C) and there was a variable amount of microtubule breakdown as well as open sheets of polymerized tubulin which appeared wider than normal microtubules (arrows, Figs. 8B and C) during incubation with nucleotides, but neither of these events correlated with the extent of 49 kDa protein released from microtubules. Although qualitative, the electron microscope observations revealed an inverse relationship between the amount of 49-kDa protein extracted by a nucleotide and the number of cross-linked microtubules remaining in a sample.

3 7. Nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled with purified 49 kDa protein and tubulin

Because nucleotide-induced disruption of crosslinking may have been affected by proteins associated with microtubules assembled in cell-free preparations, the process was reexamined by using tubulin and the 49 kDa protein purified to apparent homogeneity. Microtubules assembled from Artemia in the presence of the 49 kDa protein were collected by centrifugation through sucrose cushions, resuspended in 10 mM nucleotide solutions at room temperature for 30 min and centrifuged. Fig.9 represents typical results from such experiments. These and additional nucleotides were tested in triplicate and quantitated by scanning as described above (Fig. 10A).

Using reaction mixtures constructed from purified proteins it became possible to examine how nucleotides affected the formation of crosslinks between microtubules, an observation of special interest for nucleotides which failed to dissociate the 49 kDa protein from microtubules once crosslinking had occurred. In these experiments the nucleotides were added before the taxol-driven assembly of tubulin in the presence of the 49 kDa protein was initiated. After incubation, the microtubules were collected by centrifugation and electrophoresed, along with their corresponding supernatants on SDS polyacrylamide gels. The

Figure 9. Nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled with purified Artemia tubulin. Microtubules, assembled from purified Artemia tubulin in the presence of the 49 kDa protein (50-65% $(NH_4)_2SO_4$ fraction), were collected by centrifugation through sucrose, resuspended in 10 mM nucleotide solutions at room temperature for 30 min and centrifuged. Pellets (P) 2and supernatants (S) were resolved on SDS polyacrylamide gels and Coomassie stained. Corresponding pellets and supernatants have the same number and were the result of extractions with 1, ATP; 2, ADP; 3, AMP; 4, AMP-PNP; 5, ATP-γ-s; 6, ATP-PCP; 7, cAMP. T, tubulin; C, 49 kDa microtubule crosslinking protein. The size ($X\ 10^{-3}$) of the molecular weight markers is indicated on the right side of the figure.

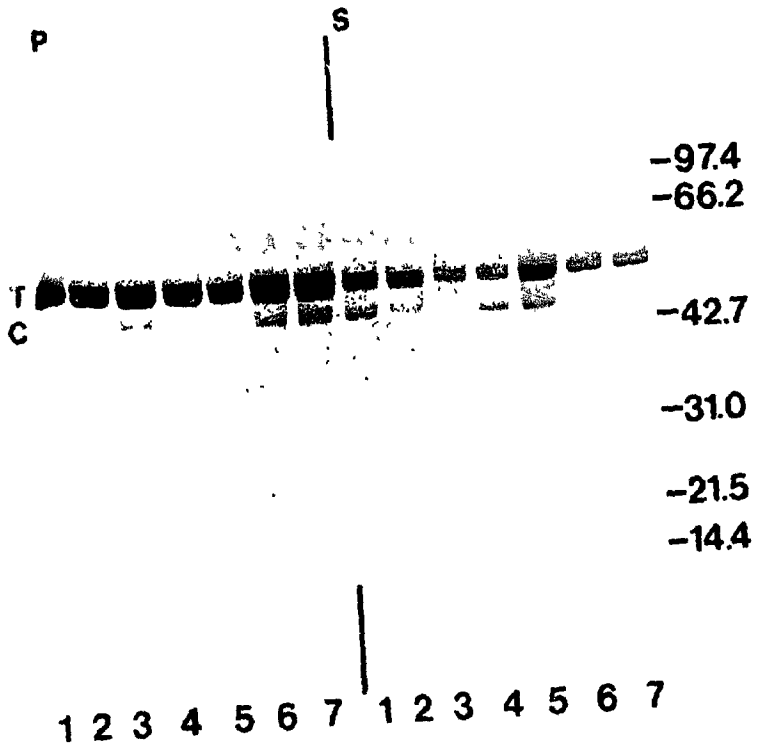

Figure 9.

Figure 10. Quantitation of nucleotide-induced dissociation of the 49 kDa protein from microtubules assembled with purified *Artemia* tubulin. The dissociation of the 49 kDa protein from microtubules by nucleotides was quantitated as described for Fig.7 except that photographs of gels instead of blots were scanned. A, nucleotides were added to microtubules after tubulin assembly in the presence of the 49 kDa protein. B, nucleotides were present during assembly of tubulin with the 49 kDa protein. Data are the mean ±S.D. of 3 experiments. The extractions were done with: 1, Pipes buffer; 2, ATP; 3, ADP; 4, AMP; 5, GTP; 6, GDP; 7, UTP; 8, CTP; 9, AMP-PNP; 10, ATP-γ-s; 11, ATP-PCP; 12, cAMP.

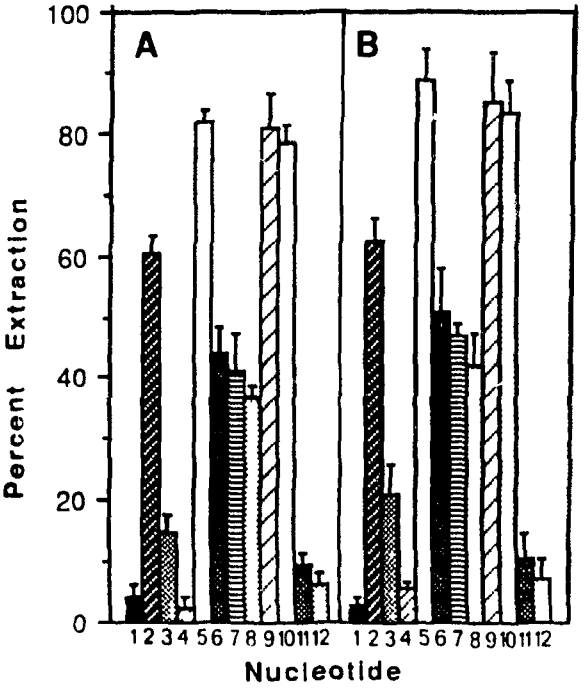
100
80
60
40
20
0
A
B
Percent Extraction
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12
Nucleotide

Figure 10.

Figure 11. Electron micrographs of microtubules assembled with purified Artemia tubulin and 49 kDa protein after exposure to nucleotide solutions. Purified tubulin was induced to assemble in the presence of the 49 kDa protein by addition of taxol. The resulting microtubules were collected by centrifugation through sucrose cushions, extracted with nucleotides, recentrifuged and prepared for electron microscopy after resuspension. A, microtubules before extraction; B and C, microtubules after extraction with ATP and GTP respectively. Note that even after incubation with GTP a small number of cross-linking particles could sometimes be found. Arrowheads, cross-linking particles; arrows, structurally abnormal tubulin polymers. The bar in A represents 0.2 μm and the magnification in all figures is the same.

Figure 11.

experiments were done in triplicate and quantitated by scanning of the gels (Fig. 10B). When comparing results with microtubules assembled in cell-free extracts (Figs 6 and 7) to those obtained when purified proteins were used (Figs. 9 and 10), only ATP (from 23% to 60%, 2.6-fold) and CTP (from 21% to 38%, 1.6-fold) gave a significant increase in the extraction of the 49 kDa protein from microtubules. Otherwise, the dissociation was about the same in both systems. Within reaction mixtures constructed from purified protein components, nucleotides tended to have a greater capacity to inhibit association of the 49 kDa protein with microtubules than to cause its removal once it had bound (Fig. 10). The effect, however, was only significant for ADP (increase from 15 to 24%; 1.6-fold) and GTP (increase from 42% to 59%; 1.4-fold), both of which were marginal. As for microtubules assembled in cell-free extracts of Artemia, electron microscopic examination revealed as inverse relationship between nucleotide extraction of the 49 kDa protein from microtubules and the number of crosslinked microtubules found on grids (Fig.11).

3.8. Nucleotidase activity of the 49 kDa protein

Fractions obtained during purification of the 49 kDa protein (Fig.1) were tested for their ability to hydrolyze ATP in either the presence or absence of microtubules Fig. 12; Table 2). As the 49 kDa protein was purified, the rate of ATP

Figure 12. Nucleotidase activity of fractions obtained during purification of the 49 kDa protein. Protein fractions were analyzed for nucleotidase activity in the absence (A) and presence (B) of microtubules as described in Materials and Methods. The fractions were: 1, S1; 2, MAPs; 3, heated MAPs; 4, P11 fraction; and 5, 50-65% $(NH_4)_2SO_4$ fraction. The curves represent the average of three determinations with S.D. shown by error bars for each point.

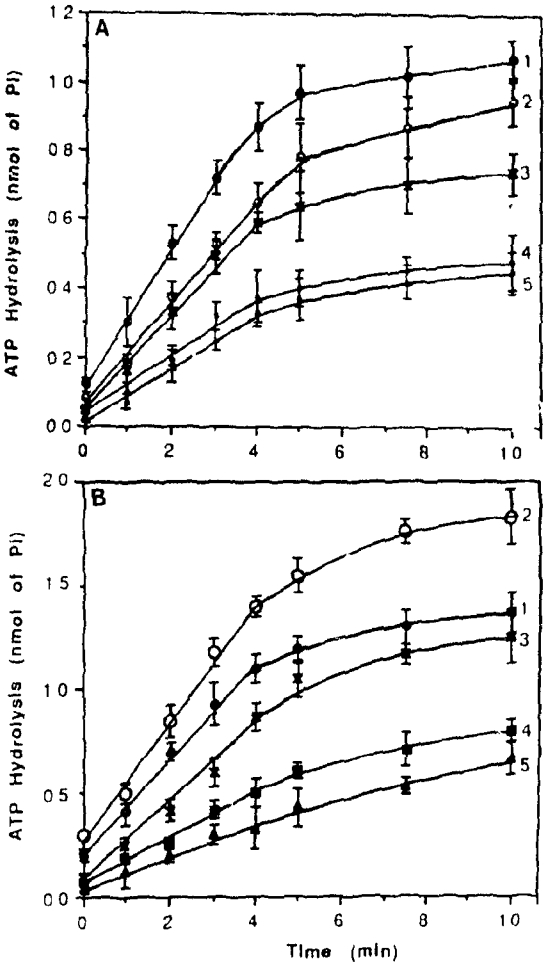
A
B
ATP Hydrolysis (nmol of Pi)
ATP Hydrolysis (nmol of Pi)
Time (min)
1
2
3
4
5

Figure 12.

hydrolysis exhibited by successive fractions in the absence of microtubules decreased from 190 to 67 nmol/min/mg, a low rate for the purified fraction. Furthermore, the recovery of nucleotidase activity was very low (Table 3) even if considered in relation to the total ATPase activity in the MAPs fraction rather than S1. Addition of microtubules to reaction mixtures yielded a small stimulation of nucleotidase activity in the MAPs and P11 fractions, but had no effect when assayed with the purified 49 kDa protein (Table 2). Including microtubules in the assays also failed to extend the 4-5 min time period over which the rate of the hydrolysis was linear (Fig. 12). ATP and GTP were superior substrates for the 49 kDa protein, both were hydrolyzed to about an equal extent and stimulation of hydrolysis by microtubules was very small for all nucleotides tested (Table 4). Nucleotidase activity of the 49 kDa protein was tested in increments of 0.5 units over the pH range of 6.8 - 8.0. For ATP, the optimal pH was 6.5, whereas for GTP the best pH was 7.0, providing a 45% increase over values obtained at pH 6.5. At each pH tested, the addition of microtubules increased nucleotide hydrolysis by an amount comparable to values in Table 4. Although not evaluated systematically, changing the concentration of the 49 kDa protein, microtubules and nucleotides within reaction mixtures had no effect on the amount or rate of nucleotide hydrolysis by the 49 kDa protein. It was clear from the results that the 49 kDa protein exhibited a very low level of hydrolysis

Table 2. ATP hydrolysis by protein purification fractions.

Fraction	Hydrolysis rate -Microtubules	Hydrolysis rate +Microtubules	Stimulation
	nmol/min/mg		%
S1	190 ± 12	175 ± 13	-
MAPs(unheated)	117 ± 15	283 ± 21	142
MAPs(heated)	100 ± 9	133 ± 23	33
P11	58 ± 8	100 ± 12	72
$(NH_4)_2SO_4$	67 ± 12	67 ± 15	-

The analysis of ATP hydrolysis rates in the presence and absence of microtubules by fractions obtained during the purification of the 49 kDa protein was described in Materials and Methods. Rates were calculated from the linear portions of the curves shown in Fig. 12 and these were obtained as the mean ± S.D. of 3 experiments. Enhancement of ATP hydrolysis rate by microtubules, when it occurred, is given as the % stimulation.

Table 3. Recovery of nucleotidase activity

Fraction	Total protein	Hydrolysis rate	Total activity	Recovery
	mg	nmol/min/mg	nmol/min	%
S1	2110.0	190	400,900	100.0
MAPs (unheated)	32.3	117	3,779	0.94
MAPs (heated)	21.3	100	2,130	0.53
P11	3.0	58	174	0.04
$(NH_4)_2SO_4$	0.4	67	27	0.007

The recovery (%) of nucleotidase activity during a typical purification of the 49 kDa protein is presented. The amount of dry Artemia cysts used as starting material was 170 g.

Table 4. Nucleotide specificity of the 49 kDa protein

Nucleotide	Hydrolysis - Microtubules	Hydrolysis + Microtubules
	nmol	nmol
ATP	1.05 ± 0.06	1.32 ± 0.10
ADP	0.38 ± 0.02	0.41 ± 0.09
AMP	0.00	0.13 ± 0.04
GTP	0.75 ± 0.06	0.83 ± 0.10
GDP	0.27 ± 0.05	0.38 ± 0.12
CTP	0.41 ± 0.06	0.45 ± 0.10
UTP	0.25 ± 0.04	0.36 ± 0.08

The 49 kDa protein, purified to apparent homogeneity, was tested in the presence and absence of microtubules for its ability to hydrolyze different nucleotides. Reactions were in Pipes buffer, pH 6.5, with the 49 kDa protein at 0.06 μg /μl, nucleotides at 1 mM and microtubules, when present, at 0.5 μg/μl. Incubations were terminated after 10 min and the amount of inorganic phosphate released (amount of hydrolysis) was determined.

ctivity, no matter which nucleotide was the substrate, and that the activity was not stimulated by microtubules.

3. 9. Characterization of the 49 kDa protein during Artemia development

The polypeptide composition of cell-free extracts prepared from Artemia at various developmental stages was relatively similar in appearance except at later stages when some prominent lower molecular weight proteins (arrowheads in Fig.13A) disappeared. Among them, an interesting protein with a molecular mass of about 25 kDa (lower arrowhead in Fig.13A) is under investigation and it may have an important physiological function. The similarity in the gel staining patterns (Fig. 13A) indicated that the proteins in the cell-free extracts were not undergoing proteolytic digestion even in later stages of development. The tubulin in equal amounts of Artemia S1 from the seven developmental stages was induced to assemble by addition of taxol. The assembled microtubules were collected by centrifugation through 15% sucrose cushions and resolved in 10% polyacrylamide gels. The SDS gels were either stained with Coomassie blue (Fig. 13B), or transferred to nitrocellulose and reacted with antibody against the 49 kDa protein (Fig.13, C). Fig. 13C reveals that the 49 kDa protein existed in all developmental stages examined.

To determine the amount of 49 kDa protein at each stage

Figure 13. Characterization of the 49 kDa protein during Artemia development. Panel A, cell-free extracts (S1) of Artemia from seven stages of development were electrophoresed on 10% SDS polyacrylamide gels and stained with Coomassie blue. Lanes 1 - 7 correspond respectively to animals developed for 0, 4, 8, 12, 17, 33 and 43 h. The sizes of the molecular weight markers on the right side of the figure are the same as described in Fig.1. Microtubule pellets assembled in cell-free extracts of Artemia corresponding in development time to those shown in panel A, were resolved on SDS polyacrylamide gels and stained with Coomassie blue (B), or blotted to nitrocellulose and immunostained with antibody raised to the amino terminal peptide of the 49 kDa protein (C). Each lane in Panel A contained 120 μg proteins while in Panels B and C, each lane contained microtubule proteins assembled from 1,200 μg of S1. Arrowheads in A, low molecular proteins which were developmentally regulated.

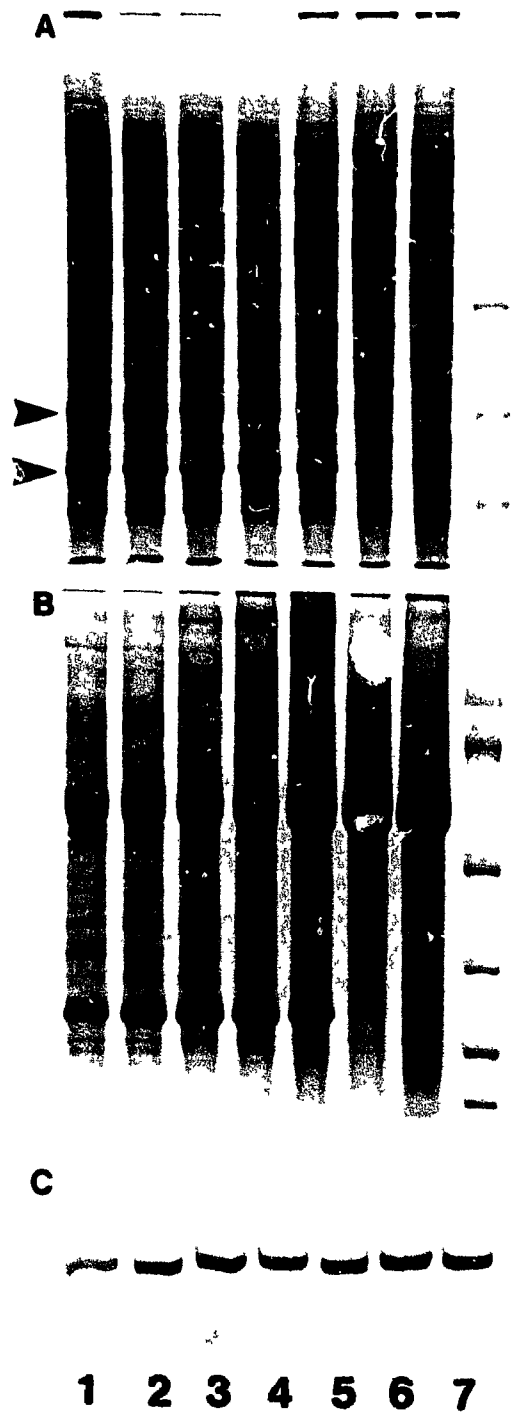

Figure 13.

Figure 14. Standard curves for quantitation of tubulin and the 49 kDa protein. In order to quantitate tubulin and the 49 kDa protein during Artemia development, standard curves were prepared. Coomassie stained gels were scanned to generate the tubulin curve (A) whereas blots were scanned to obtain the curve for the 49 kDa protein (B). The banding patterns on scanned gels (A) and blots (B) are shown in the photographic inserts. Data are the mean ±S.D. of 3 experiments.

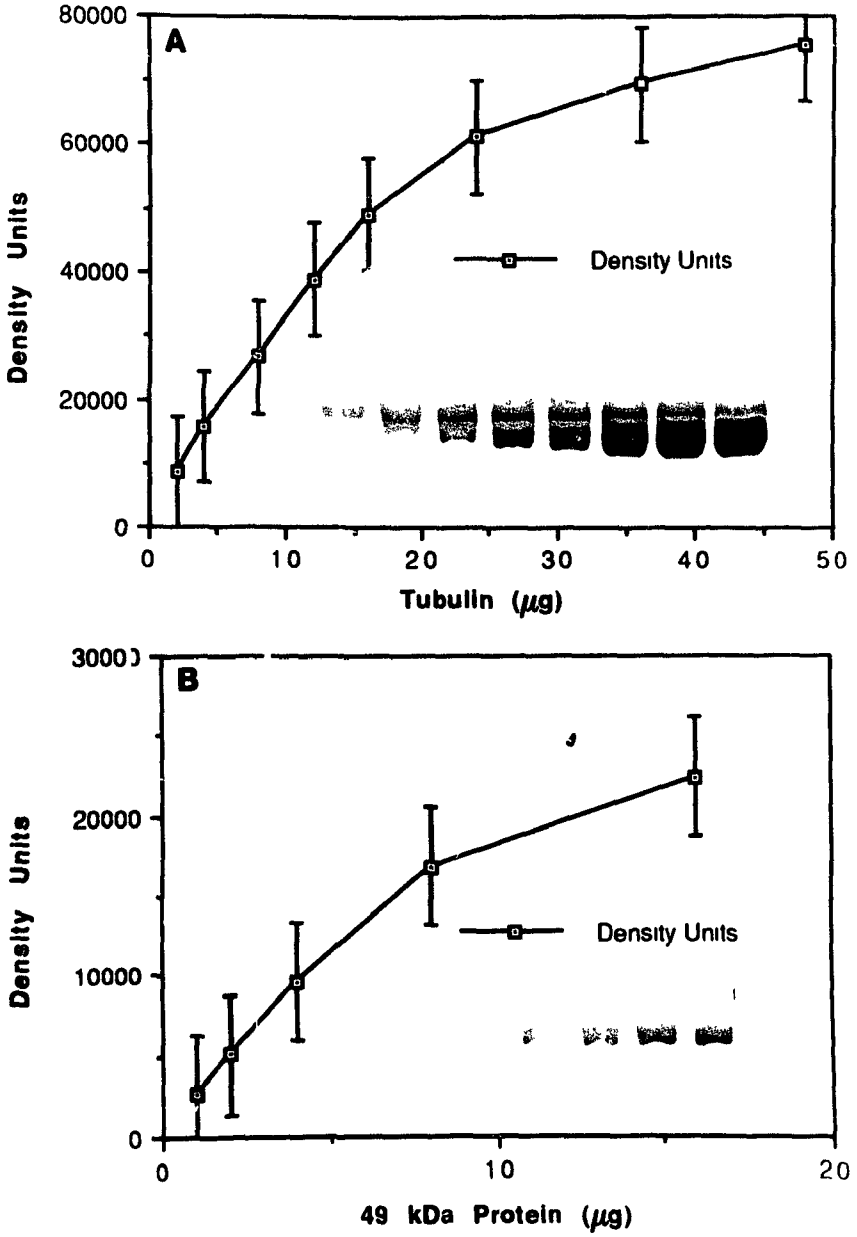
A
80000
60000
40000
20000
0
Density Units
Density Units
0
10
20
30
40
50
Tubulin (μg)
B
30000
20000
10000
0
Density Units
Density Units
0
10
20
49 kDa Protein (μg)

Figure 14.

Table 5. Quantitation of the 49 kDa protein and tubulin during *Artemia* development

Development	Arbitrary units		Ratio
Time (h)	Tubulin	49 kDa protein	Tubulin/49kD
0	25,669 ± 567	3,082 ± 231	0.12
4	26,727 ± 761	3,110 ± 202	0.12
8	27,740 ± 689	3,223 ± 154	0.12
12	29,945 ± 456	3,302 ± 303	0.11
17	31,337 ± 921	3,326 ± 239	0.11
33	32,301 ± 345	3,471 ± 167	0.11
43	32,929 ± 865	3,781 ± 359	0.11

The table contains data obtained from three repeats of experiments as described in Figs. 13 and 14. Tublin was quantitated by scanning Coomassie blue stained SDS polyacrylamide gels, while the 49 kDa protein was quantitated by scanning Western blots. Microtubules were assembled from 1,200 μg of *Artemia* S1.

of development, standard curves were established for the 49 kDa protein and tubulin (Fig.14). Tubulin in equivalent amount of cell-free extracts from each stage of development was then induced to assemble by addition of taxol, and the microtubules were collected by centrifugation through sucrose. Resulting pellets were dissolved in treatment buffer, electrophoresed on SDS polyacrylamide gels and either stained with Coomassie blue to reveal tubulin or blotted to nitrocellulose and immunostained to reveal the 49 kDa protein. Tubulin and the 49 kDa protein were then quantitated by scanning these gels (Table 5) and comparing the values obtained to the linear region of the standard curves for these proteins. The results in Table 5 were obtained by scanning three SDS polyacrylamide gels (for tubulin) and blots (for the 49 kDa protein). During early Artemia development, the amounts of tubulin and of the 49 kDa protein coassembled upon addition of taxol gradually increased, while their ratios remained almost constant.

3. 10. Immunological localization of the 49 kDa protein

3.10.1. Antibody preparation and purification

Two antibodies, one against the 49 kDa protein and the other against a peptide corresponding to the 15 amino acids of

Figure 15. Affinity purification of antibody to the 49 kDa protein. Artemia cell-free extracts (1) and MAPs (2) were blotted to nitrocellulose and immunostained. Antibodies were unfractionated serum obtained from rabbits immunized with the 49 kDa protein (A) or a peptide corresponding to its amino terminus (C) and affinity purified antibodies to the entire 49 kDa protein (B) or its amino terminus (D). Small c indicates location of the 49 kDa protein.

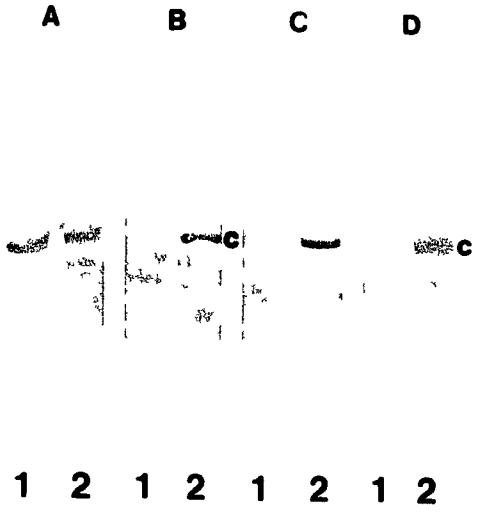

Figure 15.

its amino terminus, were prepared in rabbits and their specificity for the 49 kDa protein was tested on Western blots (Fig.15). Antibody to the 49 kDa protein was selected from serum of immunized rabbits by affinity purification on antigen-containing nitrocellulose membranes as described in Material and Methods. When tested on Western blots, the affinity purified antibody to whole protein still exhibited some nonspecific binding to proteins in cell-free supernatants from Artemia, but not to MAPs (Fig.15, panels A and B). On the other hand, affinity purified anti-peptide antibody showed no reaction with protein in cell free extracts and recognized only the 49 kDa protein in MAPs (Fig.15, panels C and D). Both of the purified antibodies were used in subsequent immunolocalization experiments with similar results, but only findings for the anti-peptide antibody are shown.

3.10.2. Recognition in vitro of microtubule-bound 49 kDa protein by affinity purified antibody

Antibodies against both the 49 kDa protein and its peptide were affinity purified for use in the immunological localization of the 49 kDa protein in Artemia larvae. To determine if these antibodies recognize 49 kDa protein bound to tubulin, crosslinked microtubules were reacted with antibodies to both tubulin (DM1A) and the 49 kDa protein. Coincident staining patterns were obtained when crosslinked microtubules were double stained, although a somewhat stronger

Figure 16. Double immunofluorescent staining of microtubules assembled from purified tubulin and 49 kDa protein. Microtubules, assembled from purified Artemia tubulin in the presence of homogeneous 49 kDa protein, were immunofluorescently stained with both DM1A, an anti-α-tubulin antibody (A) and anti-49 kDa protein antibody (B), followed by TRITC-conjugated anti-mouse IgG (A) and FITC-conjugated anti-rabbit IgG (B). The bar represents 10 μm.

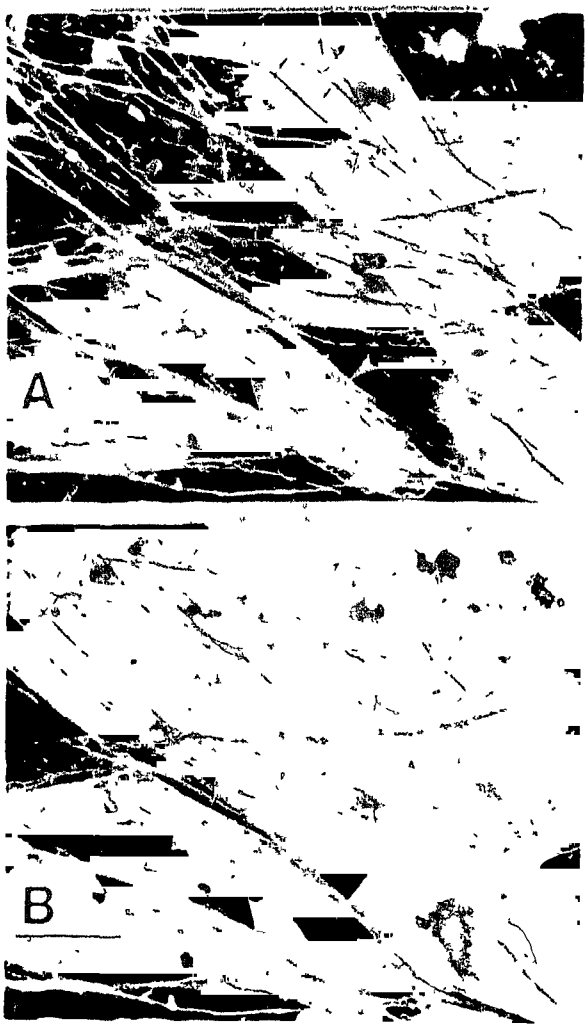

Figure 16.

Figure 17. Gold-staining of cross-linked microtubules with affinity-purified antibody to the 49 kDa protein. Microtubules, assembled from purified Artemia tubulin in the presence of 49 kDa protein, were labelled with affinity purified anti-49 kDa peptide antibody, followed with gold conjugated anti-rabbit IgG (A and B). C, no primary antibody was used. The samples were viewed with the electron microscope. The bar represents 0.1 μm.

Figure 17.

staining which appeared less grainy was observed with DM1A (Fig.16). Crosslinked microtubules were heavily labelled by the immunogold procedure (Fig.17, A and B). When the primary antibody was omitted, there was no labelling of cross-linked microtubules (Fig.17, C). Microtubules were also heavily labelled in the absence of the 49 kDa protein when application of DM1A was followed by gold-conjugated secondary antibodies to mouse IgG (not shown). Processing of gold labelled specimens obscured microtubule protofilaments and the cross-linking particles (Fig.17, A and B). Samples were thus negatively stained and examined with the electron microscope prior to antibody exposure to verify that cross-linked microtubules of normal appearance, such as those seen in Fig. 3, were present.

3.10.3. Immunofluoresence staining of Artemia Larvae

Upon demonstration of their reactivity with 49 kDa protein attached to microtubules, the affinity purified antibodies were used to fluorescently stain Artemia larvae. Mitotic spindles (arrowheads) and midbodies (arrows) were observed consistently in both single and double stained samples (Fig.18). The nuclei and condensed chromosomes sometimes fluoresced a bright orange colour (Fig.18, A-C) which revealed locations of spindles and midbodies in the absence of microtubule staining. Such observations showed that not all spindles and midbodies were stained with antibody to

Figure 18. Immunofluorescent staining of mitotic spindles and midbodies. Artemia larvae were fixed with paraformaldehyde and immunofluorescently stained. The primary antibodies were A-C, E, affinity purified antibody to the amino terminus of the 49 kDa protein; D, DM1A. Secondary antibodies were: A-C, E, FITC-conjugated anti-rabbit IgG; D, TRITC-conjugated anti-mouse IgG. D and E are the same field of view. Condensed chromosomes associated with spindles (arrowheads) and midbodies (arrows) are apparent in A-C. The bars represent 5 μm in panels A and C-E and 10 μm in panel B.

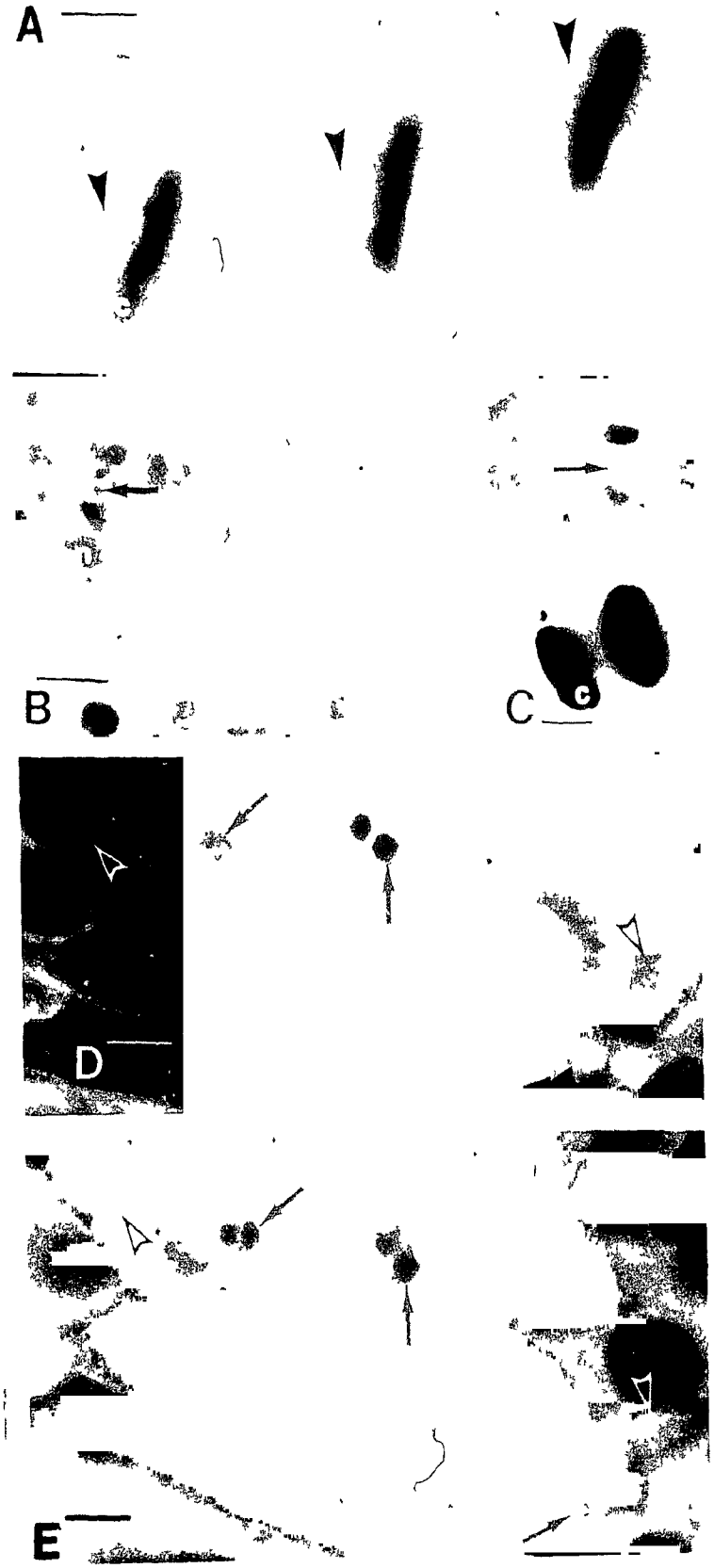

Figure 18.

Figure 19. Immunofluorescent staining of setal cells. Artemia larvae, fixed with paraformaldehyde, were immunofluorescently stained with affinity purified antibody raised to the amino terminus of the 49 kDa protein, followed by FITC-conjugated anti-rabbit IgG. The arrowheads indicate localized patches of staining along the setal cells of intermolt larvae (A). (B), Setal cells of premolt larvae in which the microtubules were withdrawn into the antenna. The arrows indicate staining along the microtubule bundles. ant, antenna. The bar represents 1 μm in A and B.

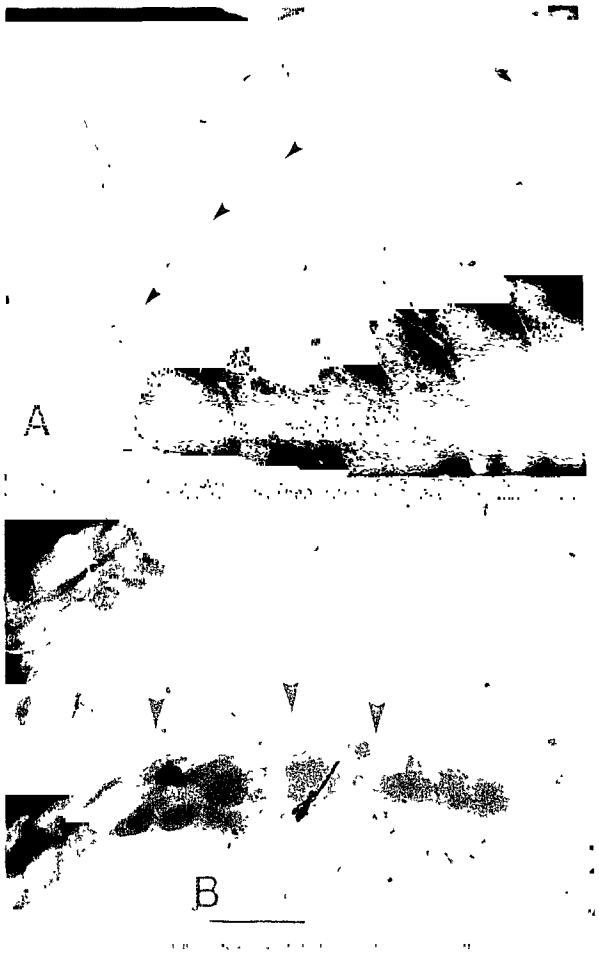

Figure 19.

the 49 kDa protein, a situation which also holds for anti-tubulin antibodies. Why this occurred is uncertain but it may indicate that the antibodies have not penetrated equally all cells. This is to be expected since the whole mount preparations used in the experiments sometimes consisted of relatively large fragments which may hinder antibody access to inner areas of the tissue. Although the staining achieved with DM1A and the anti-49 kDa antibody were colocalized, DM1A staining seemed much stronger than that obtained with the anti-49 kDa protein antibody. Moreover, when DM1A was applied, mitotic spindles and midbodies were identified frequently, while with the anti-49 kDa protein antibody, these structures were observed less often.

In addition to spindles and midbodies, affinity purified antibody to the 49 kDa protein stained microtubule bundles in the setal cells of Artemia antenna (Fig. 19). The fluorescent staining tended to occur in localized patches along the setae of intermolt larvae (Fig.19A) and it underwent rearrangement in premolt Artemia, a time when the microtubule bundles of the setal cells were withdrawn into the antenna (Fig. 19B).

3. 11. Isoform composition of the 49 kDa protein during Artemia development

Purified 49 kDa protein was resolved by isoelectric focusing into 5 bands with isoelectric points in the pH range

Figure 20. Isoform composition of purified Artemia 49 kDa protein. The purified 49 kDa protein (15 μg) was resolved into isoforms by isoelectric focusing using gels in the pH range of 4.5-7.1 (A). The portion of gel A containing the stained bands is enlarged in B. For two-dimensional analysis an isoelectric focusing gel containing the purified 49 kDa protein (15 μg) was placed on a 10% SDS-polyacrylamide gel, electrophoresed, and stained with silver (C). The isoelectric focusing gel decreased in pH from top to bottom (A), and increased from left to right (B and C).

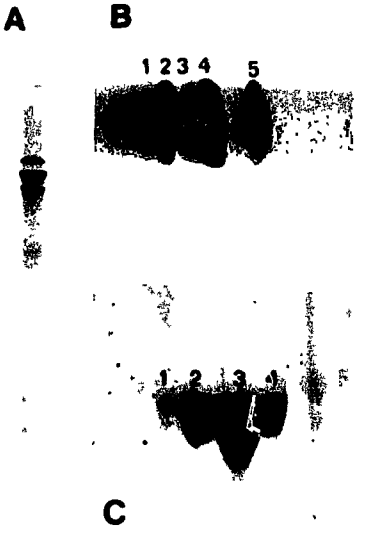

Figure 20.

Figure 21. Isoform composition of the 49 kDa protein in Artemia MAPs. The experimental procedures were the same as for Fig.21, except that the proteins analyzed were heated MAPs and the isoform patterns on two-dimensional gels was revealed by immunostaining of Western blots with antibody raised to the amino terminal peptide of the 49 kDa protein. Panel A, isoelectric focusing gel of heated MAPs; panel B, silver-stained two-dimensional gel; C, Western blot of a two-dimensional gel, immunostained with anti-49 kDa protein peptide antibody. For two dimensional electrophoresis, the acidic end of the IEF gel was to the left of the figure when overlayed on the SDS gel. Each gel contained 120 μg of MAPs.

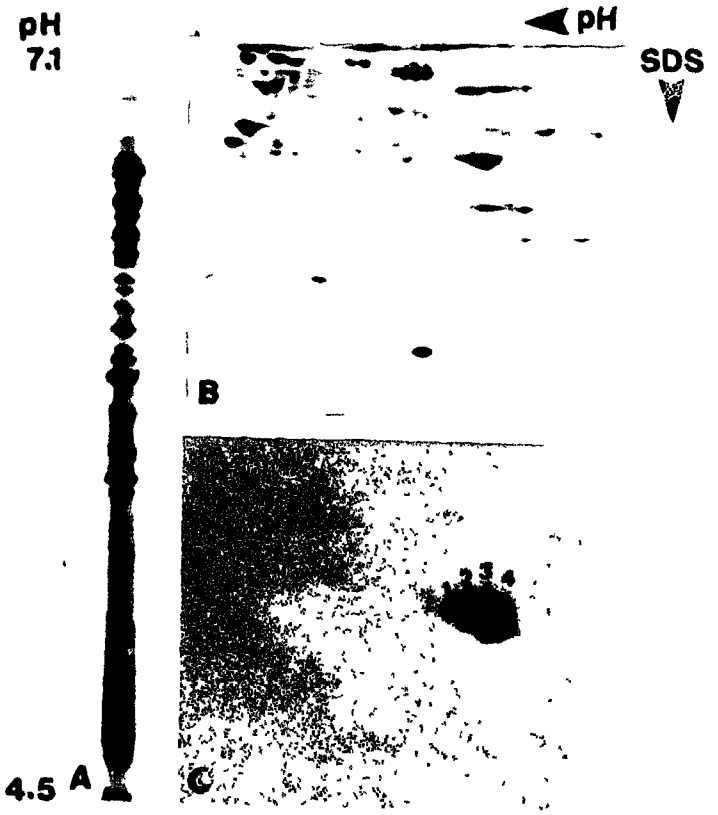

Figure 21.

6.0-6.2 (Fig. 20A). Of the five bands, three were major (labelled 2,4,5) and two were minor (labelled 1,3). When the purified 49 kDa protein was analyzed on two dimensional gels, four spots labelled 1-4 in Fig.20C were visible, presumably due to convergence of isoelectric bands 3 and 4 into the spot (labelled 3 in Fig.20C). In order to limit adventitious modification during purification and to facilitate the developmental analysis of its isoform composition, the 49 kDa protein in MAPs was examined on two-dimensional gels (Fig.21). Since the 49 kDa protein could not be recognized with certainty upon Coomassie staining (Fig.21B), the two-dimensional gels were blotted to nitrocellulose membranes and immunostained with antibody against the 49 kDa peptide (Fig. 21C). The 49 kDa protein in MAPs prepared from organisms developed for 12 h exhibited four definite isoforms (Fig.21C) as was true for silver stained gels of the purified 49 kDa protein (Fig.20C). A possible minor isoform was visible to the left of isoform 1 upon staining of blots (Fig.21C).

Further analysis was carried out to determine if the isoform composition of the 49 kDa protein varied during Artemia development. MAPs, prepared from organisms after 0, 4, 8, 12, 17, 33 and 43 h of development, were resolved by two dimensional electrophoresis, blotted to nitrocellulose and immunostained with anti-49 kDa protein antibody (Fig.22). The 49 kDa protein from various development stages consisted of at least four isoforms when resolved on two-dimensional gels.

Figure 22. Isoform composition of the 49 kDa protein during *Artemia* development. Heated MAPs, prepared after 0, 4, 8, 12, 17, 33, and 43 h of development, were electrophoresed on two-dimensional gels, blotted to nitrocellulose and immunostained with antibody to the amino terminal peptide of the 49 kDa protein. Each contained 120 μg heated MAPs. The numbers refer to the development time of the animals from which the MAPs were prepared.

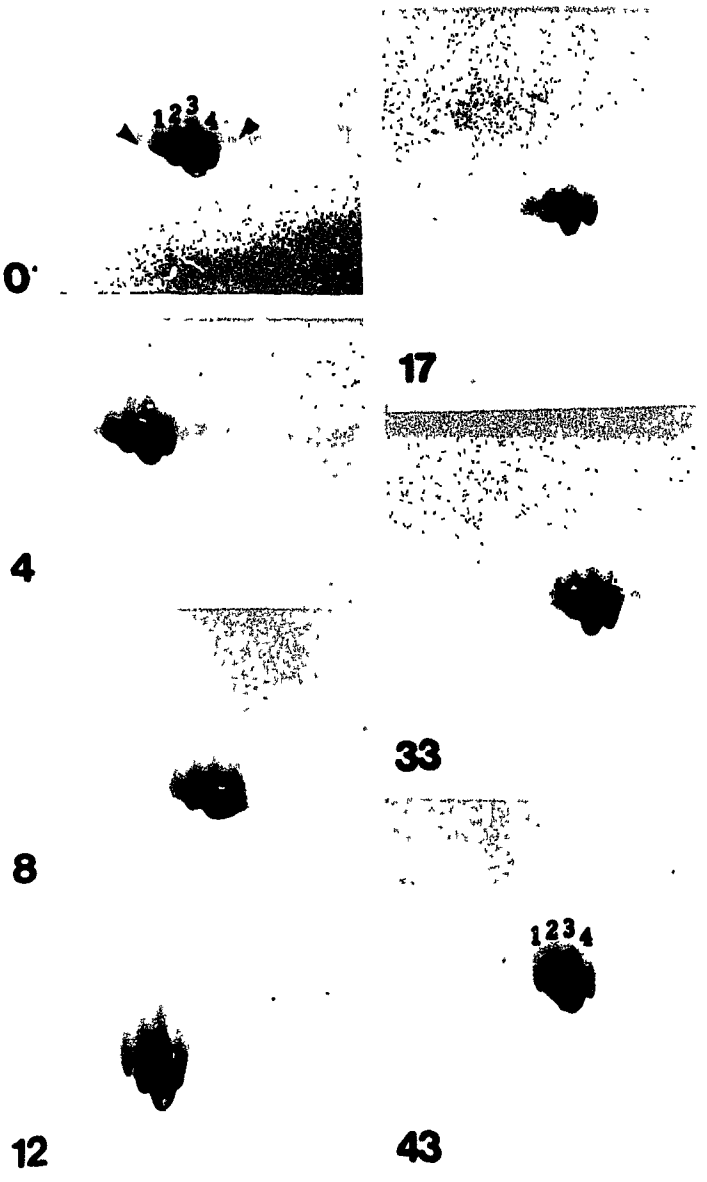

Figure 22.

These four major isoforms (numbered 1-4 in Fig.22) appeared in all developmental stages analyzed, while minor isoforms were sometimes seen (arrowheads in Fig.22), especially in samples from earlier development times. Although all major isoforms were visible for the samples from animals developed 43 h the pattern of isoforms showed slight variation from other samples (Fig.22). This may have resulted from partial digestion in spite of the presence of several proteolytic inhibitors during MAPs preparation.

3.12, Phosphorylation of the 49 kDa protein

The 49 kDa protein (0.5 μg/ul) was treated with alkaline phosphatase in a range of 25-100 units/ml, causing a downward shift in mobility of the protein on SDS gels, a response indicative of phosphate removal (Fig.23). The effect was maximal after exposure to 50 units/ml of the enzyme at 37°C for 90 min (Fig.23). To confirm the results obtained with alkaline phosphatases and to provide additional evidence for phosphorylation of the 49 kDa protein, two monoclonal antibodies, anti-phosphoserine and anti-phosphotyrosine, were used. The purified 49 kDa protein was stained by anti-phosphoserine antibody (Fig. 24A), but not by anti-phosphotyrosine antibody (Fig.24B). Moreover, when the 49 kDa protein was treated with alkaline phosphatase and immunostained with the antibodies to phosphoproteins, almost

Figure 23. Dephosphorylation of the 49 kDa protein by alkaline phosphatase. Twelve μg samples of purified 49 kDa protein were incubated with alkaline phosphatase at concentrations of 25 (lane 2), 50 (lane 3), 75 (lane 4) and 100 (lane 5) units/ml of reaction mixture in alkaline phosphatase buffer at 37°C for 90 min. At the end of the incubation, samples were boiled with an equal volume of 2X treatment buffer and then divided into two portions. One sample of about 10 μg was analyzed on 10% SDS polyacrylamide gels stained with Coomassie blue (Panel A) and the remainder (about 2 μg) was analyzed on Western blots stained with anti-49 kDa peptide antibody (Panel B). In panels A and B, lane 1 received boiled enzyme as control. Panel C, lane 1, alkaline phosphatase; lane 2, low molecular weight markers were as described in Fig.1.

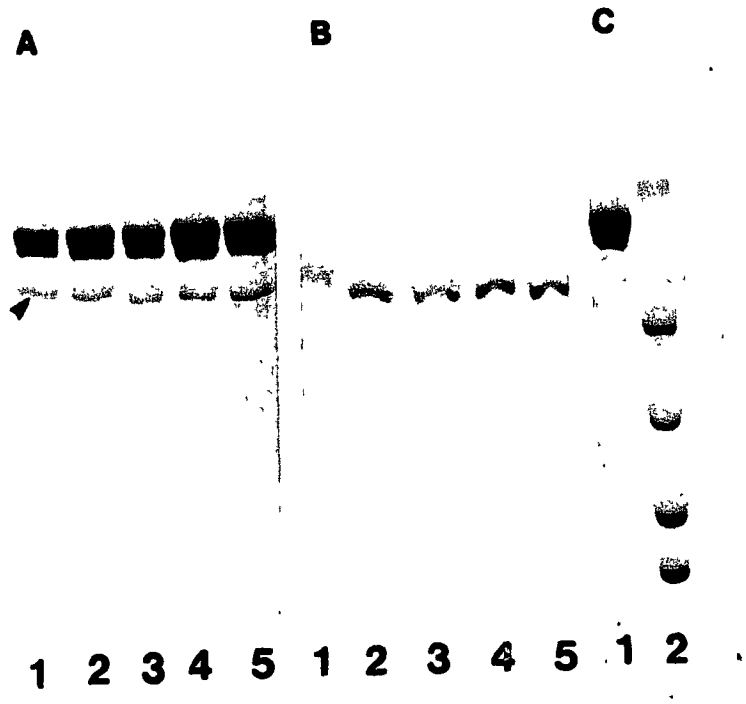

Figure 23.

Figure 24. Phosphorylation of the 49 kDa protein. Purified 49 kDa protein (12-15 μg) was resolved on 10% SDS polyacrylamide gels and either blotted to nitrocellulose and immuostained (A, B, D) or stained with Coomassie blue (C). Lane 1, purified 49 kDa protein (10 μg); lane 2, purified 49 kDa protein treated with alkaline phosphatase. The blots were immunostained with antiphosphoserine antibody (A), antiphosphotyrosine antibody (B), or secondary antibody only (D). Arrowheads show the 49 kDa protein.

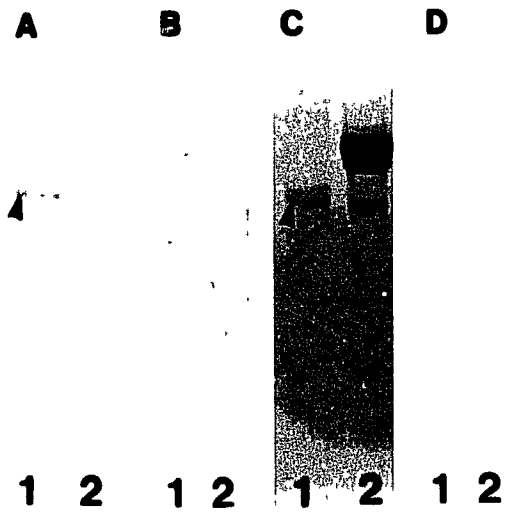

Figure 24.

Figure 25. Identification of phosphorylated isoforms of the 49 kDa protein. Isoforms of the purified 49 kDa protein were resolved by two dimensional gel electrophoresis, transferred to nitrocellulose and immuostained with either the antibody to the amino terminus of the 49 kDa protein (A and B), or anti-phosphoserine antibody (C and D). A and C contained 10 μg of protein while B and D contained 15 μg.

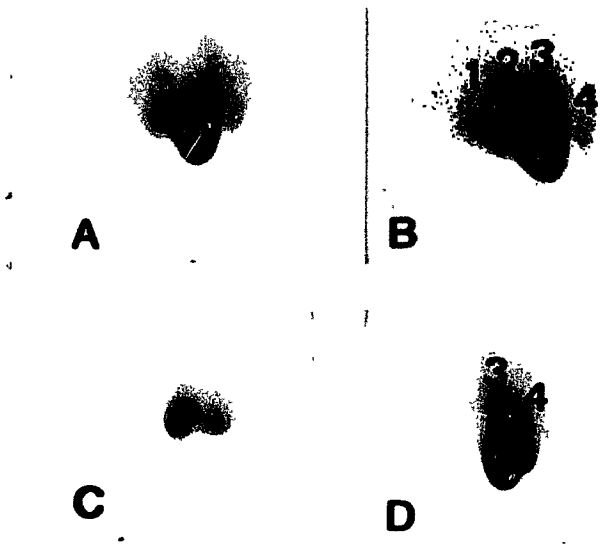

Figure 25.

Figure 26. Phosphorylation of the 49 kDa protein by cAMP-dependent kinase. The 49 kDa protein, either untreated (2) or dephosphorylated (3) with alkaline phosphatase, was incubated with cAMP-dependent kinase in the presence of γ-P^{32}-ATP at a final concentration of 0.5-1.0 mM at 37°C for 30 min. The reaction mixtures were resolved on 10% SDS polyacrylamide gels, dried and exposured to Kodak X-DMAT film overnight. Lane 1 received enzyme only. The band in lane 3 corresponding to rephosphorylated 49 kDa protein is shown by the arrow.

Figure 26.

Figure 27. Binding of dephosphorylated 49 kDa protein to microtubules in vitro. The purified 49 kDa protein was dephosphorylated by alkaline phosphatase treatment and the reaction was stopped by addition of okadaic acid, to 3 mM, followed by incubation at room temperature for 30 min. The dephosphorylated 49 kDa protein was incubated with tubulin at 1 mg/ml in the presence of 10 μM taxol at 37°C for 30 min and the coassembled microtubules were collected by centrifugation. The resulting pellets (lane 3) and supernatants (lane 4) were resolved on 10% SDS polyacrylamide gels and stained with Coomassie blue. Lane 1, untreated 49 kDa protein; lane 2, 49 kDa protein treated with alkaline phosphatase at a concentration of 150 units/ml of reaction mixture; lane 5, low molecular weight markers as described in Fig.1. T, tubulin; E, enzyme; C, 49 kDa protein.

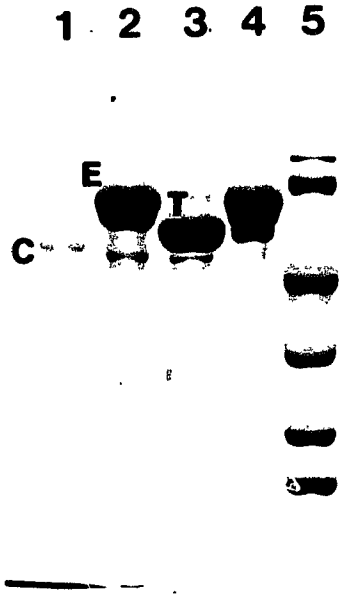

Figure 27.

no staining was observed (Fig. 24 A,B). Fig.24C shows that the 49 kDa protein used in the experiments was dephosphorylated by alkaline phosphatase while panel D was not exposed to primary antibody. Acid phosphatase did not have any effect on the 49 kDa protein (not shown), at least under my experimental conditions.

To analyze the relationship between isoform composition and phosphorylation, the 49 kDa protein was resolved on two-dimensional gels and immunostained with anti-phosphoserine antibody. At least two isoforms (labelled 3 and 4 in Fig.25) reacted with the anti-phosphoserine antibody. Since isoform 3 consists of two isoforms on two-dimensional gels, a third isoform may also be phosphorylated. When the 49 kDa protein was incubated with cAMP-dependent kinase in the presence of γ-P^{32}-ATP, phosphate groups were added only after treatment with alkaline phosphatase (Fig.26). Dephosphorylation did not affect binding of the 49 kDa protein to microtubules as shown by coassembly of tubulin and alkaline phosphatase treated 49 kDa protein (Fig. 27). Under these conditions, crosslinking appeared normal upon examination of samples with the electron microscope.

3. 13. The relationship between microfilaments and the 49 kDa protein

To determine if there is an interaction between actin and the 49 kDa protein, these proteins were incubated together

Figure 28. The 49 kDa protein does not cross-link microfilaments. Actin at 1.0 mg/ml was assembled in the presence or absence of a 50 65% $(NH_4)_2SO_4$ fraction of the 49 kDa protein and/or tubulin, as described in Materials and Methods. After centrifugation, the resulting pellets and supernatants were resolved on 10% SDS-polyacrylamide gels and stained with Coomassie blue. Lanes 1, 3, and 5 are respectively the pellets obtained by incubation of tubulin, actin and 49 kDa protein at 37°C for 30 min followed by centrifugation. Lanes 2, 4, and 6 are their corresponding supernatants. Lane 7 and 8 are the pellet and supernatant respectively from the coassembly of actin and the 49 kDa protein, indicating that the 49 kDa protein did not bind to microfilaments. Lanes 9 and 10 are the pellet and supernatant resulting from the coassembly of tubulin, actin and the 49 kDa protein. Lane 11 contained low molecular markers. Panels B and C, actin was assembled in the presence and absence respectively of the 49 kDa protein. No crosslinkers were observed among microfilaments. The bar represents 0.2 μM in B and both figures are the same magnification.

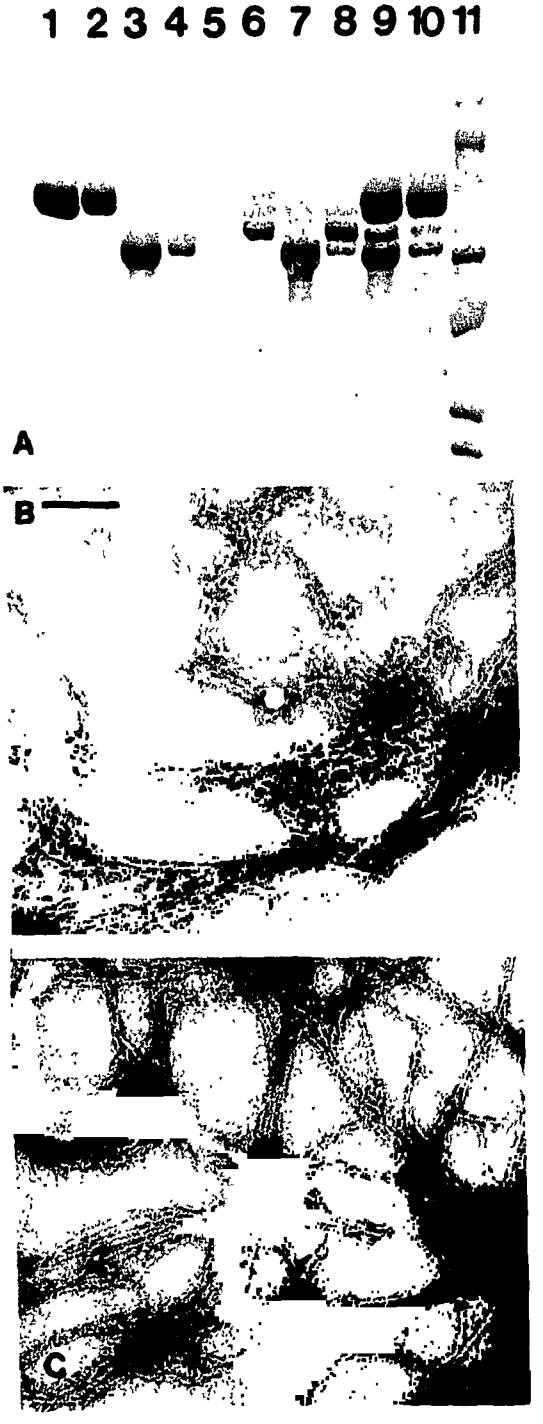

Figure 28.

under conditions which favoured their assembly. When actin and the 49 kDa protein were coassembled, microfilaments formed, but the 49 kDa protein failed to bind to these polymers and no crosslinking was observed between actin filaments (Fig.28). However, when the 49 kDa proteins were present, actin filaments seemed to be more clumped (Fig.28C). The presence of microfilaments in the reaction mixture did not interfere with binding of the 49 kDa protein to microtubules (Fig.28, lanes 9,10) an observation verified by electron microscopy.

The relative spatial distribution of actin and the 49 kDa proteins was analyzed by double staining of Artemia larvae with phalloidin, which binds to polymerized actin and the antibody to the 49 kDa protein. Salt gland cells and regions in cells adjacent to membranes were heavily stained by phalloidin, indicating they contained microfilaments, but these regions were not stained by the anti-49 kDa protein antibody. Gut muscle cells sometimes showed a weak staining with the antibody to the 49 kDa protein, but there was no correlation between the patterns of filamentous actin and the 49 kDa protein in these cells.

3.14. Survey of different species for proteins immunologically related to the 49 kDa protein

Cell-free extracts were prepared from several organisms or tissues as described in Materials and Methods and

Figure 29. Preparation of microtubule proteins from species other than Artemia. Cell-free extracts were prepared from diatoms (1), Drosophila larvae (2), snail brain (3), snail foot (4), Tilapia brain (5), and Tilapia testis (6), chicken embryos (7), bovine brain (8), and Artemia (9) as described in Materials and Methods. Panel A, cell free homogenates were electrophoresed on 10% SDS polyacrylamide gels and stained with Coomassie blue. Panel B, Taxol induced microtubules collected by centrifugation through sucrose cushions, electrophoresed on SDS polyacrylamide gels and stained with Coomassie blue. Lane 10 shows low molecular markers. Panel A, cell-free extracts (each contained 120 μg protein); Panels B, microtubule proteins assembled from 1,500 μg of cell-free extracts. T, tubulin.

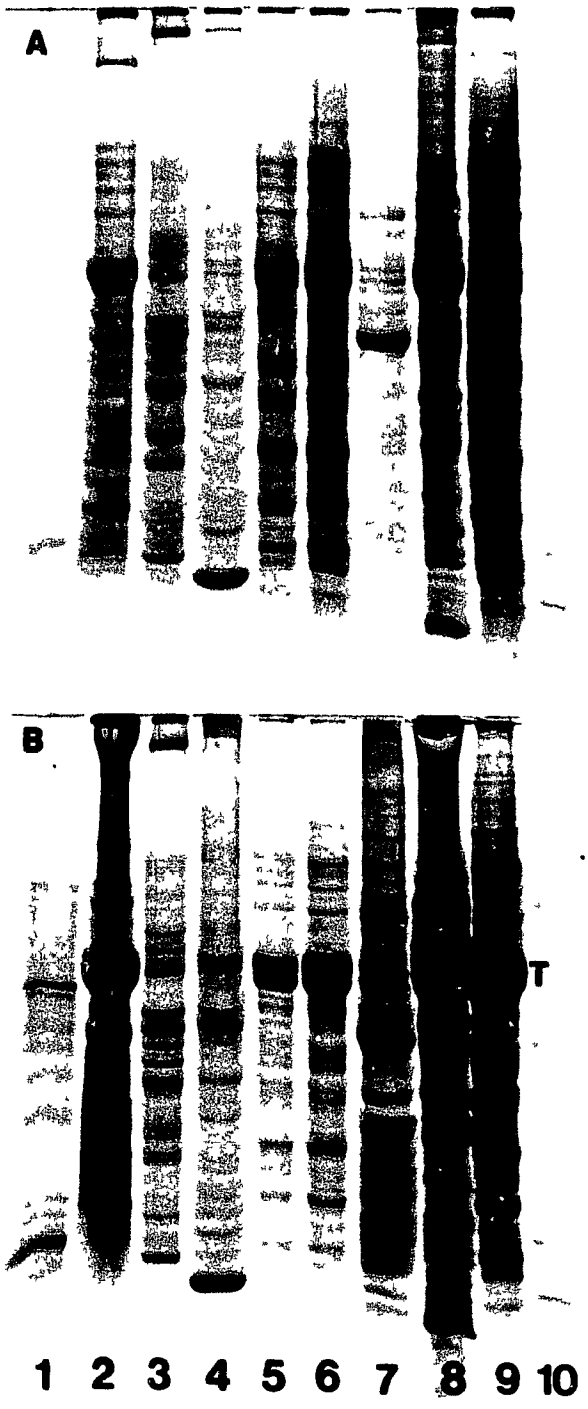

Figure 29.

electrophoresed on SDS polyacrylamide gels (Fig.29A), Microtubule proteins (tubulin and MAPs) in the cell-free supernatants were induced to assemble by addition of taxol, collected by centrifugation through sucrose cushions and either electrophoresed (Fig. 29) or examined in the electron microscope (Fig. 30). Tubulin in cell-free extracts from all organisms examined assembled into microtubules (Fig. 31). Tubulin from fruit fly larvae, fish brain and testis, chicken embryos and bovine brain polymerized readily yielding microtubules, which were relatively normal in morphology (Fig. 30). Microtubules assembled in cell-free extracts from snail were quite short and few in number (Fig.30A). No crosslinking was observed, except for Artemia microtubules.

The assembled microtubule proteins from species other than Artemia were transferred to nitrocellulose membranes after electrophoresis and reacted with antibodies. Proteins from fruit fly, snail, chicken and bovine reacted with the anti-49 kDa peptide antibody (Fig. 31A), while reactions occurred for proteins from fruit fly, and chicken embryos with the antibody raised to the entire protein (Fig. 31B). These antibodies did not react with proteins from other other species examined, such as diatoms and fish, indicating that the 49 kDa protein may not exist in these species, at least at the times examined. Although immunologically reactive proteins were present in various species, no crosslinked microtubules were observed (Fig. 30), upon examination of negatively

Figure 30. Electron micrographs of microtubules from various species. Microtubules were induced to assemble in cell-free extracts from various species and negatively stained. Figures A-F are microtubules assembled from snail, Drosophila, Tilapia brain, chicken embryos, bovine brain and Artemia respectively. Except for Artemia, no crosslinking of microtubules was observed. The bar in A represents 0.1 μm and the magnification is the same for all photographs. Arroheads show microtubule crosslinkers.

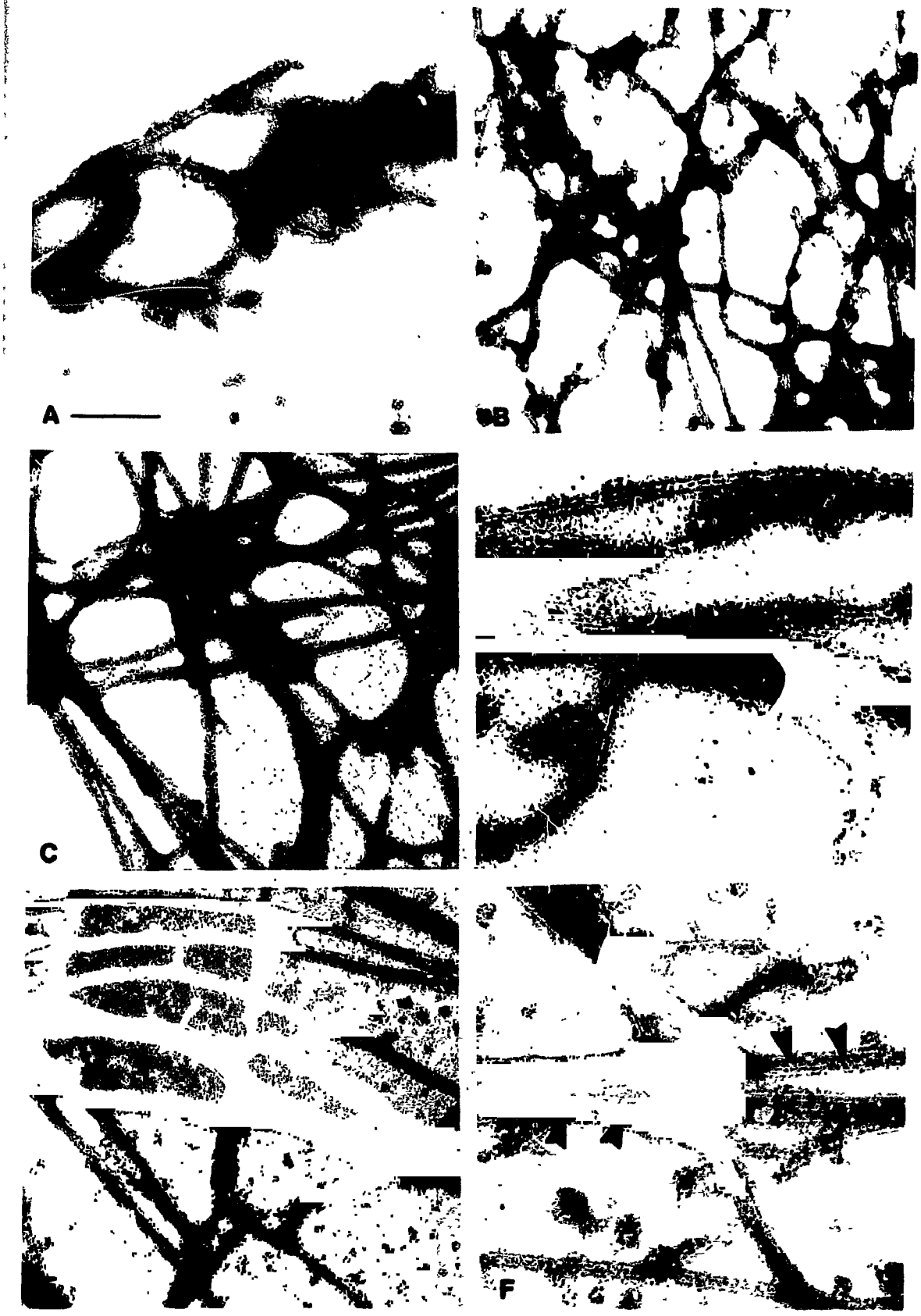

Figure 30.

Figure 31. Identification of immunological homologous of the 49 kDa protein from other species. Microtubule proteins from various species prepared as described in Figure 29 were resolved on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes and immunostained with antibody either raised to the 49 kDa peptide (panel A), or to the entire 49 kDa protein (Panel B) or with DM1A, anti-α-tubulin (Panel C). Arrowheads show cross-reacting proteins. Each lane contained microtubule proteins coassembled from 1,000 μg of cell-free extracts.

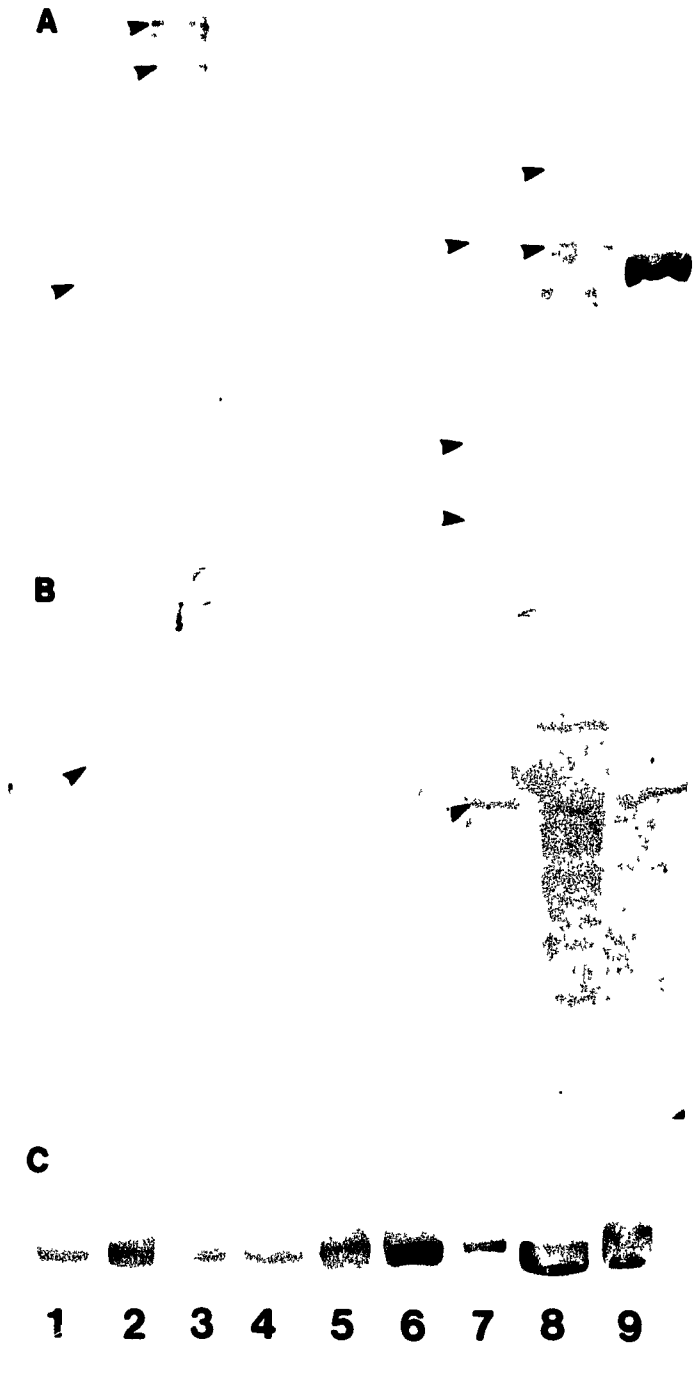

Figure 31.

stained samples with the electron microscope.

3. 15. Proteolytic digestion of the 49 kDa protein

The 49 kDa protein was digested into several fragments by papain (Fig.32) as a first step for peptide sequencing and eventual cloning of the gene for this protein. To establish optimal conditions for the digestion of the 49 kDa protein, a range of enzyme concentrations and reaction times were tested. The results revealed that the 49 kDa protein was completely digested into fragments at enzyme concentration of 0.01-0.05 μg/ml at 37°C for 20-30 min (Fig.32).

Figure 32. Proteolytic digestion of the 49 kDa protein by papain. Fifteen μg (in a 30 μl reaction mixture) of purified 49 kDa protein was incubated in the presence of 0.3 (2), 0.6 (3), 1.5 (4) and 3.0 μg (5) of papain at 37°C for 30 min. Papain at 2.0 μg each (0.06 μg/μl reaction mixture) was also incubated with the 49 kDa protein for 5 (8), 10 (9), 15 (10), 20 (11) and 30 min (12). The reactions were stopped by addition of an equal volume of 2X treatment buffer and immediate boiling. Lane 6 contained low molecular markers and lane 7 contained only papain at 2.0 μg. Lane 1, fifteen μg of the 49 kDa protein.

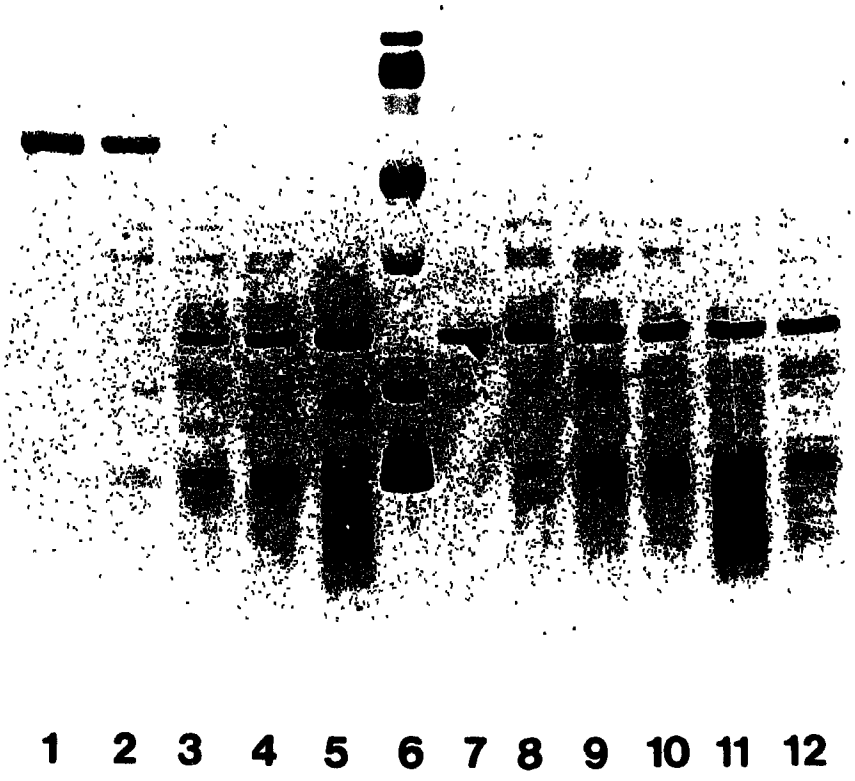

Figure 32.

IV. Discussion

4.1, Purification of the 49 kDa protein from Artemia

Campbell et al.(1989) suggested that a 49 kDa protein from Artemia functions in vitro as a microtubule crosslinker. My work, through purification of the 49 kDa protein to apparent homogeneity from cell-free extracts of Artemia and by demonstrating its coassembly with microtubules, has provided direct proof for this hypothesis. MAPs, containing the 49 kDa protein, were most efficiently extracted from microtubules by incubation for 45 min in Pipes buffer containing 0.5 M NaCl at room temperature. MAPs from Artemia are moderately heat resistant in comparison to heat stable neural MAPs, which remain functional after exposure to 100°C for 5 min (Aizawa et al. 1987). When Artemia MAPs were heated at 50°C for about 5 min, a large number of proteins precipitated and were removed by centrifugation, while the 49 kDa protein remained in solution. The 49 kDa protein resists precipitation at temperatures as high as 65°C (Fig.4), but its ability to cross-link microtubules is affected at temperature above 50°C. Addition of 10 mM DTT and 0.75 M NaCl did not increase the heat resistance of the 49 kDa protein, which is different from the results obtained for neural MAPs (Vallee 1982). The 49 kDa protein bound tightly to phosphocellulose and was eluted by Pipes buffer containing 0.2 M NaCl. Elution over such a narrow range of NaCl provided a good purification step. Final

purification was achieved by ammonium sulfate fractionation with most of the 49 kDa protein recovered in the 50-65% ammonium sulfate fraction. Some of the 49 kDa protein was lost during $(NH_4)_2SO_4$ fractionation but this was necessary to achieve complete purification, as determined by silver staining of SDS gels.

4.2, Comparison of the 49 kDa protein to other structural MAPs and its possible cellular function

One of the characteristics of structural MAPs in eukaryotic cells is that they form rodlike or granular projections on microtubules or that they link microtubules to one another, to cellular membranes or to organelles. This directly or indirectly influences cellular organization and functions (for review, see MacRae, 1992 a, b). To demonstrate that the purified 49 kDa protein is a microtubule crosslinker and that it retains activity in vitro, it was coassembled with either Artemia or bovine tubulin. The experiments revealed that the 49 kDa protein cosedimented with microtubules upon centrifugation through 15% sucrose cushions (Fig.2), and that the coassembled microtubules, observed by electron microscopy, were clearly crosslinked (Fig.3). Because the 49 kDa protein preparation used in the experiments was pure, as determined by silver staining of SDS gels, it can be concluded that the crosslinkers are formed from the 49 kDa proteins, and that other factors are not required. However, by similar

experimental procedures it was demonstrated that the 49 kDa protein did not bind to microfilaments.

The 49 kDa protein from Artemia differs from neural MAPs. Polyclonal antibodies raised to the 49 kDa protein did not recognize bovine neural MAPs on Western blots. Monoclonal antibodies to each of MAP-2 and tau tested in this study and polyclonal antibodies to MAP-2 and tau (Campbell et al., 1989) failed to react with the 49 kDa protein. The 49 kDa protein did not stimulate tubulin assembly under conditions in which neural MAPs promote tubulin polymerization (Joly et al., 1989), suggesting as one possibility that it binds to a tubulin domain different from that of neural MAPs.

Microtubule crosslinking proteins occur in nonneural cells, including proteins of 41 and 33 KDa from Crithidia (Kambadur et al., 1990; Bramblett et al., 1989), adligin from C. elegans (Aamodt et al., 1989; Aamodt and Culotti, 1986), MAPs from unfertilized sea urchin eggs (Vallee and Bloom 1983), dynamin (Scaife and Margolis, 1990; Shpetner and Vallee, 1989), a 280 kDa protein from marginal bands of toad erythrocytes (Centonze and Sloboda, 1986), and 41 and 52 kDa proteins from T. brucei (Balaban and Goldman, 1990). Based on molecular size and dissociation from microtubules by nucleotides, only the T. brucei 52 kDa microtubule crosslinking protein of those listed above is similar to the 49 kDa Artemia protein. Their similarity could be investigated further by immunological procedures using available antibodies.

The 41 kDa protein from T. brucei is aldolase, while the Crithidia 41 kDa microtubule crosslinking protein is GAPDH. The Artemia 49 kDa protein does not seem to be either of these proteins. First is the difference in molecular mass with values for GAPDH ranging from 34 to 41 kDa (Balaban and Goldman, 1990; Bramblett et al., 1989). ATP and a nonhydrolyzable analogue, AMP-PNP disrupt bundles formed with GAPDH, but the enzyme is not removed from microtubules (Huitorel and Pantaloni, 1985). The N-terminal 15 amino acids of the Artemia 49 kDa protein were determined by Edman digestion. A search of three protein databanks revealed no similarity between the amino terminus of the Artemia 49 kDa protein and this region in any other known proteins. Up to 8 of the first 15 amino acid residues of GAPDH are identical in 11 species (Michels et al., 1986), yet the Artemia 49 kDa protein does not have amino terminal residues common to GAPDH (Wierenga et al., 1987).

A 58 kDa protein from rat liver (Bloom and Brashear, 1989) and a 50 kDa protein from pig thyroid (Mithieux and Rousset, 1988, 1989) are, respectively, thought to attach Golgi and lysosomes to microtubules. The binding of the lysosome-associated protein to microtubules is disrupted by ATP independently of nucleotide hydrolysis. The 49 kDa protein does not, however, react with a monoclonal antibody (anti-58K-9) raised to the Golgi-associated microtubule binding protein (Bloom and Brashear, 1989).

How does the Artemia 49 kDa protein function in cellular organization via microtubule crosslinking? Affinity purified antibodies against either the 49 kDa protein or a peptide corresponding to the amino terminal 15 amino acid residues of the protein reacted with blots containing cell-free extracts and unheated MAPs from Artemia (Fig.15). Double staining of crosslinked microtubules with both DM1A, an anti-α-tubulin antibody, and affinity-purified anti-49 kDa peptide antibody yielded similar staining patterns, indicating that the 49 kDa protein could still be recognized by antibody after it had bound to microtubules. This conclusion was further strengthened by gold-staining, in which crosslinked microtubules were heavily labelled by the gold particles (Fig. 17). Artemia larvae were then immunofluorescently labelled with anti-49 kDa peptide antibody, revealing the cellular localization and possibly a function for this protein. The 49 kDa protein occurred in mitotic spindles (Fig. 18A), midbodies (Fig. 18B) and setal cells (Fig. 19), all of which contain organized microtubules. These structures were stained in larvae from instar I to III, but the frequency of spindle staining with the antibody to the 49 kDa is less than that obtained with anti-tubulin antibody. Perhaps the 49 kDa protein is only associated with the spindle at certain stages of mitosis. Fluorescence associated with nuclei and chomosomes (Fig. 18) was brown rather than green and thus does not represent staining with antibody to the 49 kDa protein.

Several proteins have been localized to mitotic or meiotic spindles and are thought to bundle microtubules via bridges or linkers (Bastmeyer and Fuge, 1986; Hepler at al., 1970; Izant et al., 1983). Jensen et al.(1991) suggested that the cross-linkers may have various physiological functions. They may enforce the static component of a spindle by stabilizing microtubules and protect the ends of overlapping microtubules from depolymerization, or serve as motor proteins to transduce chemical energy into mechanical force for spindle microtubule sliding. My work indicates that the 49 kDa protein has a role in structural organization of mitotic spindles. Double immunofluorescent staining using anti-tubulin and anti-49 kDa protein antibodies revealed the presence of the 49 kDa protein in spindles (Fig.18, D and E). Whether the 49 kDa protein functions as a motor able to drive spindle microtubule sliding can only be determined by motility assays employing video microscopy. However, the low nucleotidase activity exhibited by the 49 kDa protein in either the presence or absence of microtubules and under the conditions where the 49 kDa protein is removed from microtubules by nucleotides, suggests against this possibility. Masson and Kreis (1993) reported that a protein, E-MAP-115, identified by screening a HeLa cDNA library, modulates the polarization and differentiation of epithilial cells by affecting microtubule organization. Whether the 49 kDa protein has this function in Artemia remains uncertain. Setal cells from Artemia were

frequently stained by the anti-49 kDa protein antibody (Fig. 19). As these specialized cells contain organized microtubules, the 49 kDa protein may influence their spatial arrangement.

4.3. The nucleotide sensitivity and nucleotidase activity of the 49 kDa protein

Dissociation of the 49 kDa protein from microtubules is nucleotide specific and, as judged by the response to AMP-PNP, independent of nucleotide hydrolysis. The effects of ATP and CTP are influenced by the relative purity of the 49 kDa protein preparation employed in the assays with separation of the 49 kDa protein from microtubules less effective when microtubules are assembled in cell-free preparations than when purified proteins are used. Microtubules formed in cell-free extracts, even collected through sucrose cushions, have many proteins associated with them. An ATP-specific nucleotidase may copurify with the polymerized tubulin and hydrolyze ATP sufficiently and rapidly so that it does not disrupt crosslinking. This would not be expected in reaction mixtures containing only tubulin and the 49 kDa protein. That ATP hydrolysis happens in the nonpurified fraction is indirectly supported by the observation that ADP and AMP are relatively ineffective in removing the 49 kDa protein from microtubules. On the other hand, the analysis of nucleotidase activity indicates that only a limited amount of ATP is hydrolysed in

a MAPs preparation, whether or not microtubules are present. Thus, if ATP destroys microtubule crosslinking a sufficient amount should remain, in spite of enzymatic activity, to allow this to occur. Two analogues of ATP, namely AMP-PNP and ATP-γ-s dissociate the 49 kDa protein from microtubules under all conditions tested, whereas AMP-PCP does not. That dissociation when it happens is due to ATP contamination of the analogues is unlikely since this nucleotide does not efficiently separate the 49 kDa protein from microtubules assembled in cell-free extracts, but both AMP-PNP and ATP-γ-s are effective under these circumstances. The results indicate that nucleotide hydrolysis is not necessary for separation of the 49 kDa protein from microtubules. However the purified 49 kDa protein does exhibit a low nucleotidase activity which prefers ATP and GTP as substrates and is not stimulated by microtubules.

How do nucleotides exert their effects on microtubule crosslinking? A trivial explanation for the results is that nucleotides modify the pH or ionic strength causing dissociation of the 49 kDa protein from microtubules. Contrary to this possibility is that no change in pH is detected in Pipes buffer upon addition of nucleotides to 10 mM, an amount used commonly by others in these types of experiments. Efficient separation of the 49 kDa protein from microtubules requires NaCl concentrations in excess of 300 mM, suggesting that nucleotides at 10 mM are apt to be insignificant from an

ionic perspective. Moreover, if these effects were ionic, then all nucleotides would be expected to have a similar impact on microtubule crosslinking, but this is not the case. Since there is almost a complete dissociation of the 49 kDa protein from microtubules for some of the nucleotides, a direct interaction of the nucleotide with the 49 kDa protein is indicated, perhaps causing a conformational change in the molecule. This could lead to breakdown of the 49 kDa protein aggregates, which from the size of interconnecting particles seen with the electron microscope, appear to be the arrangement of the protein active in microtubule organization. Attempts to bind ATP and GTP to the 49 kDa protein, either in solution or on blots were not successful, although other proteins bound GTP under these conditions (not shown). Also, the 49 kDa protein did not adhere to columns consisting of ATP and GTP attached to crosslinked 4% beaded agarose (Sigma Chemical Co.). another possible effect of nucleotides, considered unlikely, is that they change tubulin structure in a way that inhibits the binding of the 49 kDa protein to microtubules.

The nucleotide responses of dynamic MAPs have been particularly well studied and they provide interesting contrasts to the 49 kDa microtubule crosslinker from Artemia. Stimulation of kinesin ATPase activity by microtubules varies from 2- to 1000-fold (Hackney, 1988; Shimizu et al., 1991) and occurs even when the C-terminal domain of tubulin is removed

by subtilisin (Rodionov et al.,1990). At least one kinesin-like protein bundles microtubules (Andrews et al., 1993; Nislow et al., 1992). The bundles are disrupted by ATP but not by AMP-PNP, an analogue which causes kinesin to bind tightly to microtubules and is used in the purification of this protein (Vale et al.,1985). Cytoplasmic dynein is an ATPase extracted from microtubules by ATP but not GTP (Paschal and Vallee, 1987; Paschal et al., 1987; McIntosh and Pfarr, 1990). The ATPase of cytoplasmic dyneins, with at least one exception (Grissom et al., 1992), is activated by microtubules and the specific activities range from 0-850 nmol/min/mg of protein (Hollenbeck et al., 1984; Paschal et al., 1987; Schroeder et al., 1990). Cytoplasmic dynein from brain, among other samples, hydrolyses GTP, TTP and CTP more readily than ATP, although microtubule stimulation is minor for nucleotides other than ATP (Shpetner et al., 1988). Microtubules are bundled by cytoplasmic dynein from several sources (Hollenbeck and Chapman, 1986; Amos 1989) and in all cases save one, ATP disrupts the bundles. However, for one of these proteins, termed HMW4, 50% of the protein remains associated with the microtubules even though the bundling is almost completely eliminated by ATP (Hollenbeck and Chapman, 1986). Also, in the study where it was examined, microtubule bundles were not destroyed by AMP-PNP or AMP-γ-s. Enzymatic studies revealed that a dynamin GTPase activity is stimulated up to 150-fold by microtubules (Shpetner and Vallee, 1989). Maeda et al.(1992)

report only partial extraction of dynamin from microtubules by GTP, whereas ATP-γ-s, GDP and GMP-PNP had little effect. Movement of bundled microtubules upon exposure to GTP was not observed.

As judged by its sensitivity to nucleotides, the 49 kDa protein could be a dynamic MAP (Chapin and Bulinski, 1992). However, how does the 49 kDa protein relate to other dynamic MAPs, such as kinesin and cytoplasmic dynein? Kinesin and kinesin-like proteins were reported to be involved in mitosis and meiosis (Heck et al., 1993; Changdra et al., 1993; Wright et al., 1993; Nislow et al.,1992; Sawin et al.,1992). In Drosophila, a kinesin -like protein, named KLP61F, functions in spindle pole separation and mitotic spindle dynamics (Heck et al., 1993). In Aspergillus nidulans, bimCKLP is necessary for the separation of duplicated spindle pole bodies (Enos and Morries, 1990). The CENP-E centrosome protein is a kinesin-like protein that is required for the progression from metaphase to anaphase (Yen et al., 1992) and the Xenopus Eg5 gene encodes a protein that is important for bipolar spindle assembly (Roof et al., 1992). Intermicrotubule bridges in mitotic spindles have been reported (Jensen et al., 1991 a.b.), but there is only a limited amount of structural evidence to indicate that dynamic MAPs physically crosslink microtubules in spindles (Andrews et al., 1993; Hirokawa et al.,1988). Aother dynamic MAP, cytoplasmic dynein, is also required for normal nuclear segregation in yeast (Eshel et

al., 1993). The 49 kDa protein exists in spindles and midbodies, suggesting an organizational or physiological role in cell division (Fig. 18) and it exhibits a limited nucleotidase activity. It thus shares properties of both structural and dynamic MAPs and it is premature to classify the 49 kDa protein in either group based on current evidence.

4.4. The developmental expression of the 49 kDa protein and its isoform composition

The 49 kDa protein was present at all stages of development examined in this study. As summarized in Table 5, both tubulin and the 49 kDa protein increase slightly during early development, while the ratio of the 49 kDa protein to tubulin is almost constant. Moreover, the amount of 49 kDa protein recovered by taxol-induced assembly in relation to the total amount of protein in cell-free extracts at each stage increases somewhat during development.

In order to further characterize the 49 kDa protein it was subjected to isoelectric focusing revealing that it consisted of 5 isoforms with pIs in the range of 6.0-6.2. Modification of the pH profile by using other ratios of ampholytes did not yield an increase in the number of isoforms visible on isoelectric focusing gels. On the two dimensional gels, the 49 kDa protein clearly showed four isoforms in both purified (Fig.20) and partially purified (Fig.21) protein fractions. The isoforms labelled 3 and 4 on isoelectric

focusing gels are thought to have merged during electrophoresis in the second dimension. As the isoform composition of some MAPs, such as MAP2 and tau, are developmentally regulated (Wille et al., 1992), the 49 kDa protein was tested for this possibility. MAPs from seven development stages at 0, 4, 8, 12, 17, 33 and 43 h were blotted to nitrocellulose and probed with the anti-49 kDa peptide antibody. The major isoforms were present at all developmental stages, and in some stages potential minor isoforms were observed (Fig.22, arrowheads). At the moment, I am not sure if the minor isoforms are real or the result of limited proteolytic digestion. The latter case is unlikely because inhibitors of proteolytic enzymes were added to each preparation and the experiments were repeated at least three time with similar results.

In comparison to other MAPs, the synthesis and isoform composition of MAP2 and tau proteins have been well studied. MAP2 has three isoforms, termed MAP2a, 2b and 2c, which are formed by posttranscriptional processing of mRNA derived from a single MAP2 gene (Garner and Matus, 1988). MAP2c is abundant in immature or newborn brains, and disappears in adults. MAP2b remains relatively constant during brain development, while MAP2a gradually increases and reaches a maximum during early growth (see reviews, Vallee, 1990; MacRae, 1992 a, b). Another neural MAP termed tau consists of several polypeptides with molecular masses in the range of 55-62 kDa (Drubin and

Kirschner, 1986). In newborn rat brain, only two tau polypeptides appear, while 12 day old brain contains five different taus (Ginzburg et al., 1982). In chicken brain, the smallest tau (~50 kDa) increases rapidly for the first two days of development, and then gradually decrease thereafter (Binder et al., 1984). My results reveal that the amount of 49 kDa protein and its isoform composition are relatively constant during early development. Such consistency suggests that each of the 49 kDa isoforms has functional significance during Artemia development.

The 49 kDa protein in Artemia consists of five isoforms. How are these isoforms generated ? Are they products of different genes or do they arise by posttranslational modification? Multiple isoforms of tubulin in most eukaryotes are produced, for example, by expression of different members of small gene family and by posttanslational modifications (MacRae and Langdon, 1989). The functional significance of the tubulin isoforms remains uncertain (Langdon et al., 1991 a,b). As described earlier, tau consists of at least six isoforms and they are believed to be formed, at least in part, by phosphorylation (Butler and Shelanski, 1986). My work confirms that the isoforms of the 49 kDa protein are derived in part by phosphorylation, a posttranslational modification. The 49 kDa protein is labelled by antiphosphoserine antibody on Western blots (Fig. 23, panel B, lane 1) and on 2-D gels, two isoforms were stained with the antibody (Fig. 25). Upon treatment with

alkaline phosphatase, the 49 kDa protein undergoes a change in motility on SDS polyacrylamide gels (Fig. 24), a modification indicative of dephosphorylation (Hisanaga et al., 1993, 1989; Ulloa et al., 1993). when the 49 kDa protein was treated with alkaline phosphatase and then reacted with antiphosphoserine antibody on Western blots, almost no staining occurred (Fig. 23, panel B, lane 2). Additionally, the 49 kDa protein was only rephosphorylated by cAMP-dependent kinase after treatment by alkaline phosphatase (Fig. 26). However, we cannot exclude the possibility that some or all of the isoforms of the 49 kDa protein are derived from different genes, or by posttranscriptional processing of mRNA. This question can only be answered by molecular approaches such as gene cloning and sequencing.

Most MAPs, once they are phosphorylated, lose their ability to bind microtubules. Examples include a 70 kDa protein from HeLa cells (Rickard and Kreis, 1991), MAP4 (Aizawa et al., 1991) and kinesin (Yoshitake et al., 1992). Under our experiment conditions, however, the dephosphorylated 49 kDa protein binds microtubules in vitro (Fig. 27); This could be explained as for tau protein by Ancos et al. (1993) whereby phosphorylation at certain sites is needed for tau binding to microtubules and phosphorylation at other sites may prevent this association. The results suggest that phosphorylation does not occur at the microtubule binding sites in the 49 kDa protein and that phosphorylation and

dephosphorylation are not involved in the regulation of the 49 kDa protein in binding to microtubules, at least *in vitro*.

Tubulin was induced to assemble in cell-free extracts of various species by addition of taxol and except for diatoms microtubules of normal morphology were obtained. However, no microtubule crosslinking was observed for species other than *Artemia*. Polyclonal antibodies raised against either the entire 49 kDa protein or its amino terminal peptide cross-reacted with a polypeptide of approximately 50 kDa associated with microtubules from *Drosophila* and chicken embryos (Fig. 31). For snail and bovine, reactions only occurred with the the antibody raised to the 49 kDa peptide antibody. There was, however, relatively heavy background staining of lanes containing bovine microtubule proteins putting this result in doubt. Also, for snail, the cross-reacting proteins were much higher in molecular mass than 49 kDa. The results suggest that the 49 kDa protein exists in early developmental stages from some species other than *Artemia*. The relationships between these proteins will not be precisely defined until their amino acid sequences are known.

4.5, Conclusions

As proposed by Campbell *et al.*(1989), a 49 kDa protein from *Artemia* crosslinks microtubules *in vitro*, possibly also *in vivo*. The 49 kDa protein exhibits a set of novel characteristics. It is moderately resistant to heat, it does

not stimulate tubulin polymerization and it is immunologically different from neural MAPs and kinesin. The sensitivity of the 49 kDa protein to nucleotides in microtubule crosslinking suggests a physiological role for nucleotides in the regulation of its function. The 49 kDa protein exhibits a low nucleotidase activity not stimulated by microtubules under the assay conditions examined. It is, to the best of my knowledge, the first MAP to show a nucleotidase activity insensitive to stimulation by microtubules. The relatively constant amount of the 49 kDa protein and of its isoform composition indicate that this protein functions at all development stages. The 49 kDa protein isoforms may be generated in part, by phosphorylation, a posttranslational modification, but the number of genes for this protein in Artemia remains uncertain. Immunofluorescent staining of Artemia larvae with anti-49 kDa protein antibody revealed that the protein occurs in mitotic spindles, midbodies, and setal cells, all of which contain organized arrays of microtubules, suggesting a role in spatial arrangement of microtubules. Actin filaments exist in all eukaryotic nonmuscle cells as one of three components of the cytoskeleton, but the 49 kDa protein does not crosslink microfilaments. Affinity-purified anti-49 kDa peptide antibody was used to survey a variety of cells and tissues for the presence of antigenically related polypeptides. Cross-reacting polypeptides were found in microtubule protein preparations from fruit fly, snail, and chicken embryos, but no

crosslinkers were observed between their microtubules. The overall data support the proposal that a novel family of proteins with the ability to modulate microtubule organization in a nucleotide-dependent manner exists in Artemia.

Appendix 1

Solutions and Recipes

1. Solutions used in tubulin preparation

1.1. Pipes buffer: 100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA. (Adjust pH to 6.5 with 10 N NaOH)

1.2. Pipes + 0.2 M NaCl: Pipes, $MgCl_2$, and EGTA as in 1.1. 0.2 M NaCl, pH 6.5

1.3. Pipes + 0.3 M NaCl: Pipes, $MgCl_2$ and EGTA as in 1.1. 0.3 M NaCl, pH 6.5

1.4. Pipes + 1.0 M NaCl: Pipes, $MgCl_2$ and EGTA as in 1.1. 1.0 M NaCl, pH 6.5

1.5. Pipes + 4 M glycerol: Pipes, $MgCl_2$ and EGTA as in 1.1. 4 M glycerol, pH 6.5

1.6. Pipes + 8 M glycerol: Pipes, $MgCl_2$ and EGTA as in 1.1. 8 M glycerol, pH 6.5

1.7. 100 mM $MgCl_2$ in Pipes buffer: dissolve 203 mg $MgCl_2$ in 10 ml of Pipes buffer

1.8. 18 mM GTP in Pipes buffer: dissolve 94 mg GTP in 10 ml of Pipes buffer

1.9. Hatch medium:

NaCl	148.2 g
$MgSO_4.7H_2O$	37.6 g
$MgCl_2.6H_2O$	27.2 g
KCl	4.2 g
$CaCl_2.2H_2O$	1.3 g
$NaHCO_3$	0.2 g
Na borate	6.0 g
Add water to 6 L	

1.10. Proteolytic inhibitors:
Solution A: Leupeptin 10 mg, Soybean trypsin inhibitor 10 mg, dissolve in 10 ml of Pipes buffer
Solution B: Pepstatin A 5 mg, PMSF 10 mg, dissolve in 5 ml of ethanol.

2. Solutions for SDS-PAGE

1A: acrylamide 39.0 g

Bis 1.0 g
glycerol 20.0 ml
add water to 100 ml, stir and filter

1B: Tris 18.3 g
20% SDS 2.5 ml
add water to 100 ml, stir and filter, pH 8.8

1C: TEMED 0.1 ml in 50 ml of water

1D: Ammonium persulfate: 50 mg in 20 ml of water

2B: Tris 6.0 g
20% SDS 2.5 ml
add water to 100 ml, stir and filter, ph 6.8

2C: TEMED 0.2 ml in 10 ml of water

Electrode buffer: Tris 12.0 g
glycine 57.6 g
20% SDS 8 ml
dH_2O to 4 L

Staining solution: water 1060 ml
Coomassie Blue 10 g
acetic acid 140 ml
methanol 800 ml
add water to 2 L, stir for about 1 hr, then filter through miracloth

Destaining solution: methanol 400 ml
acetic acid 140 ml
glycerol 100 ml
add water to 2 L, filter

4x Treatment buffer: 0.125 M Tris 0.60 g
4% SDS 1.60 g
20%glycerol 8.0 ml
10% 2-ME 4.0 ml
add water to 20 ml, pH 6.8

1% bromophenol blue: 1.0 g bromophenol blue in 100 ml of water

3. Reagents for Lowry method

Solution A: 2% sodium carbonate solution 100 ml
2% Na K tartrate solution 1 ml
1% cupric sulfate solution 1 ml
mix just before use

Solution B: dilute the Folin reagent 1:2 with water

4. Solutions for Western blotting

4.1. Blotting buffer:
25 mM Tris
192 mM glycine
20% (v/v) methanol

4.2. 5X TBS stocking solution:
50 mM Tris-HCl (pH7.4)
700 mM NaCl

4.3. TBS-Tween 20:
5XTBS 200 ml
0.1% Tween 20 1 ml
add water to 1 L

4.4. HST buffer:
10 mM Tris-HCL (ph 7.4)
1 M NaCl
0.5% (v/v)Tween 20

4.5. Substrate solution:
BCIP 82.5 µl
NBT 165.0 µl
in 25 ml of AP buffer

4.6. AP buffer:
100 mM Tris
100 mM NaCl
5 mM $MgCl_2.6H_2O$
pH 9.5

4.7. Stop solution:
20 mM Tris
5 mM EDTA
pH 8.0

4.8. Blocking solution: 5% milk or bovine serum albumin

5. Solutions for 2-D gels

5.1. Isoelectric focusing gel:
2.75 g urea (Fisher)
0.5 ml 38% acrylamide
1.0 ml 10% NP40
1.25 ml dH_2O
After urea was dissolved, 0.5 ml of Bio-lyte ampholytes 3/10 (Bio-Rad) was added. The solution was then deaerated and polymerized by the addition of 3.5 µl of TEMED and 5 µl of 10% ammonium persulfate.

5.2. IEF overlay solution:
2.75 g urea
200 µl Bio-lyte 5/7 ampholyte
50 µl Bio-lyte 3/10 ampholyte
dH_2O to 5 ml

5.3. IEF anode buffer
(10 mM) 2.04 ml phosphoric acid in 3 L dH_2O

25.4. IEF gel fixative
4.0% sulfosalicylic acid
12.5% trichloroacetic acid

5.5. IEF gel staining solution
27% (v/v) isopropanol
10% (v/v) acetic acid
0.04% Coomassie blue R-250
0.5% $CuSO_4$, added last to avoid precipitation

5.6. IEF gel destaining 1
12% (v/v) isopropanol
7% (v/v) acetic acid
0.5% $CuSO_4$

5.7. IEF gel destaining 2
7% acetic acid
5% methanol

5.8. 2-D PAGE denaturing solution
12.5% (v/v) SDS-PAGE stacking gel buffer
1.5% SDS
10% glycerol
5% mercaptoethanol

6. Buffers for dephosphorylation

6.1. Alkaline phosphatase buffer
20 mM Tri-HCl (pH 8.5)
150 mM NaCl
2 mM $NaCl_2$
0.1 mM EGTA
0.1 mM EDTA

6.2. Acid phosphatase buffer
10 mM Pipes (pH 6.5)
0.1 M NaCl
1 mM $MgCl_2$
0.1 mM EGTA
0.1 mM EDTA

7. Others

7.1. 250 μM taxol:
5 μl 250 mM taxol stock in DMSO
95 μl DMSO
100 μl Pipes buffer, pH 6.5

7.2. 15% sucrose cushion
100 mM Pipes buffer, pH 6.5
4 M glycerol
15% sucrose

7.3. Low molecular markers
5 μl of LMW stock solution
45 μl of 2X Pipes buffer
50 μl of 2X treatment bufier

7.4. PBS (pH 7.4)

N2aCl	8.0 g
KCl	0.2 g
$Na_2HPO_4.7H_2O$	2.1 g
KH_2PO_4	0.2 g

Add dH_2O to 1000 ml

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