Influence of phosphorylation on isoform composition and function of a microtubule-associated protein from developing *Artemia*

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A novel 49 kDa protein, which exhibits nucleotide-dependent cross-linking of microtubules *in vitro* and localizes to ordered microtubule arrays by immunofluorescent staining, has been purified to apparent homogeneity from the brine shrimp, *Artemia*. Electrophoretic analysis involving isoelectric focusing and two-dimensional gels, supplemented by staining of Western blots with affinity-purified antibody, revealed that the 49 kDa protein consists of five isoforms with pI values of 6.0–6.2. The amount of 49 kDa protein increased slightly, but its isoform composition did not change significantly, during development of *Artemia* gastrula to third-instar larvae. Treatment with alkaline phosphatase caused the 49 kDa protein to undergo a mobility shift on gel electrophoresis, and, by use of an antibody to phosphoserine,

at least two isoforms of the protein were shown to be phosphorylated. The serine phosphate, presumably added by a post-translational mechanism, did not influence binding of the 49 kDa protein to microtubules. Under conditions in which microtubules were cross-linked, the 49 kDa protein failed to interact with actin filaments. Our results demonstrate that the 49 kDa protein, like other structural microtubule-associated proteins such as tau and MAP2, is composed of several isoforms, some of which are phosphorylated. This protein has the potential to regulate the spatial distribution of microtubules within cells but does not link microfilaments to one another or to microtubules.

INTRODUCTION

Microtubules, which exist in eukaryotic cells as one of three principal filamentous elements of the cytoskeleton, are assembled from tubulin, a heterodimeric protein formed of α - and β -subunits [1–4]. Many proteins, known as microtubule-associated proteins (MAPs), interact with microtubules and are copurified with tubulin during temperature-dependent cycles of assembly and disassembly. The interplay of MAPs and microtubules influences the related cell properties of shape, polarity and cytoplasmic organization [2,5–13]. As the physiological functions of MAPs prepared from both neural and non-neural sources became known, they were divided into two types, the architectural or structural and the energy-transducing or dynamic [2,14–16]. The first group of MAPs either modulates tubulin polymerization or its members link microtubules to one another or to other organelles, with both of these activities involved in the extension of cell processes and the establishment of morphology. The second group includes the microtubule-based mechanochemical enzymes, such as kinesin, kinesin-like proteins and cytoplasmic dynein. Their role is to direct movement of subcellular components, including chromosomes and membrane-bound vesicles, tasks accomplished by the hydrolysis of either ATP or other nucleotides.

Recently, several new MAPs exhibiting unusual characteristics when compared with the well-studied neural MAP have been found. For example, Masson and Kreis [17] described a protein, E-MAP-115, expressed predominantly in epithelial cells and thought to function in their polarization. A 77 kDa protein, abundant in echinoderms, may mediate microtubule–ribosome attachment [18]. A 97 kDa protein, possibly part of a larger

complex with ATP-sensitive binding to microtubules, occurs at the tips of cilia [19] and MAPs of about 65 kDa are observed in bovine pancreas [20] and tobacco BY-2 cells [21].

Many MAPs bundle or cross-link microtubules [5,14,21–28], a property of importance for cytoplasmic organization. We have isolated a 49 kDa MAP from the brine shrimp, *Artemia* [15,16,26], which cross-links microtubules in a nucleotide-dependent fashion. The protein possesses a modest nucleotidase activity insensitive to stimulation by microtubules and it localizes to ordered microtubule arrays. To reveal its characteristics further, the isoform composition and phosphorylation of the 49 kDa protein, properties with functional significance for MAPs such as tau and MAP2, were analysed. Moreover, we wished to know if the 49 kDa protein interacted with cytoskeletal elements other than microtubules, thus providing more evidence of its activities *in vivo*. The results of these studies are presented in this paper.

MATERIALS AND METHODS

General procedures

Artemia franciscana, obtained from Sanders Brine Shrimp Co. (Ogden, UT, U.S.A.) as encysted gastrula, were hydrated and washed with cold water, then grown in hatch medium. Larvae were synchronized at instar I by phototaxis [29,30]. Purification of tubulin and the 49 kDa protein, taxol-induced assembly of tubulin in cell-free extracts and NaCl-dependent extraction of MAPs, SDS/PAGE, isoelectric focusing (IEF), two-dimensional gel electrophoresis, Western blotting to nitrocellulose, staining of blots with either affinity-purified antibody raised to an N-terminal peptide of the 49 kDa protein or antibodies to phosphoproteins

Abbreviations used: MAP, microtubule-associated protein; IEF, isoelectric focusing.

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(Sigma, St. Louis, MO, U.S.A.), scanning of gels and blots with an Abaton 300-GS scanner (Everex Systems, Fremont, CA, U.S.A.) and examination of negatively stained samples with the electron microscope were as described previously [15,16,26,29,30]. Taxol was generously given by Dr. Nancita R. Lomax (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.). Chemicals were obtained from Sigma, ICN Biomedicals (St. Laurent, Quebec, Canada) or BDH (Dartmouth, Nova Scotia, Canada) and were reagent-grade or better.

Dephosphorylation of the 49 kDa protein

Dephosphorylation reactions were essentially as described by Hisanaga et al. [31]. Samples containing purified 49 kDa protein at 0.3–0.5 $\mu\text{g}/\text{ml}$ were incubated at 37 °C for 90 min with alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1) (Sigma) dissolved in 20 mM Tris/HCl, pH 8.5, containing 150 mM NaCl, 2 mM MgCl_2 , 0.1 mM EGTA and 0.1 mM EDTA and used at the concentrations stated in the Figure legends. The final pH varied somewhat, depending on the volume of 49 kDa protein, dissolved in Pipes buffer, which was added to the reaction mixture. The dephosphorylation was performed in the presence of proteolytic enzyme inhibitors including leupeptin and soybean trypsin inhibitor at 20 $\mu\text{g}/\text{ml}$, pepstatin A at 25 $\mu\text{g}/\text{ml}$ and phenylmethane sulphonyl fluoride at 50 $\mu\text{g}/\text{ml}$. After incubation with alkaline phosphatase, samples were heated in treatment buffer, electrophoresed and either stained with Coomassie Blue or blotted to nitrocellulose and allowed to react with antibody. For microtubule-binding assays, dephosphorylated samples of the 49 kDa protein were incubated with 3 mM okadaic acid (Sigma) for 30 min at room temperature. Purified *Artemia* tubulin at 1 mg/ml, taxol at 10 μM and GTP at 1.8 mM were then added to reaction mixtures which were incubated at 37 °C for 30 min before centrifugation at 40000 g at 20 °C. The resulting pellets and supernatants were analysed by SDS/PAGE. Samples were removed from assembly mixtures before centrifugation, negatively stained and examined in the electron microscope.

Incubation of actin with the 49 kDa protein

Monomeric actin from bovine muscle (Sigma) was incubated at 37 °C for 30 min in Pipes buffer containing MgCl_2 at 8 mM, to which was added as indicated in the Figure legend, purified 49 kDa protein to 0.5 mg/ml, purified *Artemia* tubulin at 1 mg/ml and taxol to 10 μM . The assembly reaction mixtures were centrifuged at 40000 g for 30 min at 20 °C, and the resulting pellets and supernatants analysed by SDS/PAGE. Samples were taken before centrifugation, negatively stained and examined in the electron microscope.

RESULTS

Occurrence of the 49 kDa protein in developing *Artemia*

The polypeptide composition of cell-free extracts prepared from *Artemia* at different developmental stages was similar except for the disappearance of some prominent low-molecular-mass proteins as incubation time increased (Figure 1a). The similarity in the gel-staining patterns indicated that the proteins in cell-free extracts were not undergoing proteolytic digestion even in later stages of development. As the 49 kDa protein could not be detected in cell-free extracts [15], tubulin was induced to assemble by addition of taxol. The microtubules were collected through sucrose, resolved in SDS/polyacrylamide gels and either stained

Figure 1. Occurrence of the 49 kDa protein in developing *Artemia*

(a) Cell-free extracts obtained from *Artemia* after 0, 4, 8, 12, 17, 33 and 43 h of development (lanes 1–7 respectively) were electrophoresed in SDS/10% polyacrylamide gels and stained with Coomassie Blue. Each lane received 120 μg of protein. Arrowheads indicate low-molecular-mass proteins reduced in amount during development. (b) and (c) Microtubule pellets assembled in cell-free extracts corresponding to those in (a) were resolved in SDS/polyacrylamide gels and either stained with Coomassie Blue (b) or blotted to nitrocellulose and stained with antibody to the 49 kDa protein (c). Each lane contained microtubule proteins assembled from 1200 μg of cell-free extract protein. Lane 8, molecular-mass markers (kDa). T, tubulin.

with Coomassie Blue (Figure 1b) or blotted to nitrocellulose and allowed to react with antibody (Figure 1c), revealing the 49 kDa protein at all developmental stages examined.

Quantification of the 49 kDa protein

For quantification, gels and blots prepared in triplicate as just described (Figure 1) were scanned with a densitometer and the values obtained for the 49 kDa protein and tubulin were compared with the linear portions of standard curves established for both of these proteins. To prepare standard curves, purified tubulin (α - and β -subunits) (0–50 $\mu\text{g}/\text{lane}$) and 49 kDa protein (0–20 $\mu\text{g}/\text{lane}$) were electrophoresed on SDS/10% polyacrylamide gels. The gels were stained with Coomassie Blue to reveal tubulin whereas for the 49 kDa protein the gels were blotted and immunostained before scanning. Curves were then constructed by plotting the densitometer values obtained (arbitrary units) against the quantity of tubulin in each lane. The ratio of 49 kDa protein to tubulin ($\mu\text{g}/\mu\text{g}$) declined slightly during development

Table 1 Quantification of the 49 kDa protein

The 49 kDa protein and tubulin were quantified during *Artemia* development. At each time period microtubules were assembled from 1200 μg of cell-free extract protein. The amounts of 49 kDa protein and tubulin were calculated from the linear portions of standard curves which were obtained as the mean $\pm$ S.D. of three experiments.

Development time (h)	49 kDa protein (μg)	Tubulin (μg)	Ratio of 49 kDa protein:tubulin ($\mu\text{g}/\mu\text{g}$)
0	1.2 ± 0.1	7.7 ± 0.2	0.16:1
4	1.2 ± 0.1	8.0 ± 0.2	0.15:1
8	1.3 ± 0.1	8.3 ± 0.2	0.16:1
12	1.3 ± 0.1	9.0 ± 0.1	0.14:1
17	1.3 ± 0.1	9.4 ± 0.3	0.14:1
33	1.4 ± 0.1	9.7 ± 0.1	0.14:1
44	1.4 ± 0.1	9.9 ± 0.3	0.14:1

Figure 2 Isoform composition of the purified 49 kDa protein

(a) Purified 49 kDa protein (15 μg) was electrophoresed in IEF gels, which had a pH profile from top to bottom of 7.1 to 4.5, and stained with Coomassie Blue. (b) The portion of the gel shown in (a) that contained the 49 kDa protein was enlarged to display the isoforms, numbered 1–5, more clearly. (c) Purified 49 kDa protein (15 μg) was electrophoresed in a two-dimensional gel and stained with silver to reveal isoforms numbered 1–4. The directions of IEF and SDS/PAGE in this and subsequent Figures are indicated by arrowheads labelled pH and SDS respectively.

and there was a gradual increase in the amount of each protein (Table 1). Because it was possible that the microtubules were saturated by an excess of 49 kDa protein, purified *Artemia* tubulin was added to cell-free supernatants which had undergone one round of taxol-induced assembly and the polymerization was repeated. These microtubules were collected through sucrose, electrophoresed and either stained with Coomassie Blue or blotted to nitrocellulose and allowed to react with antibody. Results like those shown in Figures 1(b) and 1(c) were obtained for the first assembly, whereas only a few proteins in addition to tubulin were visible after the second round of polymerization. None of these proteins reacted with antibody to the 49 kDa protein even though colour development in the alkaline

Figure 3 Isoform composition of the 49 kDa protein in MAP preparations

(a) Heated *Artemia* MAPs were electrophoresed in IEF gels with a pH range of 7.1–4.5 and stained with Coomassie Blue. (b) Silver-stained two-dimensional gel of heated *Artemia* MAPs. (c) Western blot from a two-dimensional gel of heated MAPs allowed to react with antibody to the 49 kDa protein and showing spots numbered 1–4. Only a portion of the gel is shown explaining the larger size of the spots in (c) compared with (b). For two-dimensional electrophoresis the acidic end of the IEF gel was to the left side of the SDS/polyacrylamide gel. Each gel received 120 μg of protein.

phosphatase substrate was sufficiently long that non-specifically stained tubulin began to appear (not shown). Clearly, all of the 49 kDa protein in each sample of cell-free extract was recovered in the first taxol-induced assembly.

Isoform composition of the 49 kDa protein

Purified 49 kDa protein was resolved by IEF into five bands with isoelectric points in the pH range 6.0–6.2 (Figures 2a and 2b). Of the five bands, three were major (labelled 2, 4 and 5 in Figures 2a and 2b) and two were minor (labelled 1 and 3 in Figures 2a and 2b). Analysis on two-dimensional gels, however, yielded four spots, presumably because of convergence of isoelectric bands 3 and 4 into the spot labelled 3 (Figure 2c). In order to limit the possibility of adventitious modification and to facilitate developmental analysis of its isoform composition, the 49 kDa protein in MAPs prepared in the presence of proteolytic enzyme inhibitors was examined in two-dimensional gels. As the 49 kDa protein could not be recognized with certainty by Coomassie staining (Figures 3a and 3b), although a strong candidate is visible in Figure 3b (arrowhead), two-dimensional gels were blotted to nitrocellulose and allowed to react with antibody. Four spots (Figure 3c), as seen on silver-stained gels of the purified 49 kDa protein (Figure 2c), were observed, as was a minor spot to the left of isoform 1 in Figure 3(c).

The 49 kDa protein in MAPs from different developmental times consisted of at least four spots when examined in two-dimensional gels (Figures 4a–4g) and minor spots were sometimes seen (arrowheads). For animals grown for 43 h, the pattern varied slightly although all four major spots were visible on blots (Figure 4g). This may have resulted from partial digestion in spite of the inclusion of proteolytic enzyme inhibitors.

Phosphorylation of the 49 kDa protein

The 49 kDa protein was exposed to alkaline phosphatase, causing it to undergo a downward mobility shift in SDS/polyacrylamide

Figure 4 Isoform composition of the 49 kDa protein during *Artemia* development

Heated MAPs, prepared from *Artemia* developed for 0, 4, 8, 12, 17, 33 and 43 h (**a–g** respectively) were electrophoresed in two-dimensional gels, blotted to nitrocellulose and stained with antibody to the 49 kDa protein. Each gel received 120 μ g of protein and only the region containing the 49 kDa protein is shown. The major spots are numbered 1–4 in (**a**) and (**g**), and representative minor isoforms are indicated in some panels by unlabelled arrowheads.

Figure 5 Gel mobility shift of the 49 kDa protein after exposure to alkaline phosphatase

Purified 49 kDa protein (12 μ g) was incubated at 37 °C for 90 min with alkaline phosphatase at 25, 50, 75 and 100 units/ml of reaction mixture (lanes 2–5 respectively in **a** and **b**), then heated in a boiling water bath with an equal volume of 2 $\times$ electrophoresis treatment buffer. Then 10 μ g from each sample was analysed by SDS/PAGE with Coomassie Blue staining (**a**) and the remaining 2 μ g was electrophoresed, blotted to nitrocellulose and stained with antibody to the 49 kDa protein (**b**). As control, the 49 kDa protein in lane 1 was exposed to boiled enzyme. (**c**) Lane 1, alkaline phosphatase; lane 2, molecular-mass markers as shown in Figure 1(a). Arrowhead, 49 kDa protein.

gels, a response indicative of phosphate removal (Figure 5). The effect was maximal after exposure to 50 units/ml enzyme at 37 °C for 90 min. As additional evidence for phosphorylation a monoclonal antibody to phosphoserine recognized the purified 49 kDa protein blotted to nitrocellulose, although an antibody to phosphotyrosine did not (Figure 6). Antibodies to phosphoserine and phosphotyrosine were obtained from Sigma. When the 49 kDa protein was treated with alkaline phosphatase, interaction with the anti-phosphoserine antibody was eliminated (Figure 6a). However, dephosphorylation under these conditions

Figure 6 Immunological analysis of 49 kDa protein phosphorylation

Purified 49 kDa protein was resolved in SDS/polyacrylamide gels and either stained with Coomassie Blue to show the phosphatase-induced mobility shift (**c**) or blotted to nitrocellulose and allowed to react with antibody to phosphoserine (**a**), phosphotyrosine (**b**) or secondary antibody only (**d**). Lane 1, purified 49 kDa protein; lane 2, purified 49 kDa protein treated with 100 units/ml alkaline phosphatase. Arrowheads, 49 kDa protein.

Figure 7 Binding of dephosphorylated 49 kDa protein to microtubules *in vitro*

Purified 49 kDa protein was exposed to alkaline phosphatase at 150 units/ml and the reaction was stopped by incubation with 3 mM okadaic acid at room temperature for 30 min. The dephosphorylated 49 kDa protein was incubated at 37 °C for 30 min with *Artemia* tubulin at 1 mg/ml and taxol at 10 μ M, followed by centrifugation to collect microtubules. Lane 1, 49 kDa protein; lane 2, 49 kDa protein treated with alkaline phosphatase; lane 3, microtubule pellet; lane 4, supernatant from centrifugation; lane 5, molecular-mass markers shown in Figure 1(a). C, 49 kDa protein; E, alkaline phosphatase; T, tubulin.

Figure 8 Identification of phosphorylated 49 kDa protein isoforms

Isoforms of the purified 49 kDa protein were resolved by two-dimensional gel electrophoresis, transferred to nitrocellulose and stained with antibody to either the 49 kDa protein (**a** and **b**) or to phosphoserine (**c** and **d**). (**a**) and (**c**) received 10 μ g of protein and (**b**) and (**d**) received 15 μ g.

Figure 9 Microfilaments and the 49 kDa protein

(a) Actin at 1.0 mg/ml was assembled at 37 °C for 30 min in the presence or absence of purified 49 kDa protein and/or *Artemia* tubulin. After centrifugation, the resulting pellets and supernatants were resolved in SDS/polyacrylamide gels and stained with Coomassie Blue. Lanes 1, 3 and 5 are respectively the pellets obtained by centrifugation after incubation of tubulin, actin and 49 kDa protein separate from one another, and lanes 2, 4 and 6 are their corresponding supernatants. Lanes 7 and 8 are the pellet and supernatant respectively after co-incubation of actin and the 49 kDa protein followed by centrifugation. Lanes 9 and 10 are the pellet and supernatant respectively obtained by centrifugation after co-incubation of tubulin, actin and the 49 kDa protein. Lane 11 contains molecular-mass markers as shown in Figure 1(a). t, tubulin; a, actin; c, 49 kDa protein. (b) and (c) Electron micrographs of negatively stained microfilaments obtained on assembly of actin in the presence and absence respectively of the 49 kDa protein. Arrowheads, microfilaments. The bar in (b) represents 0.2 μ m and both Figures are the same magnification.

did not prevent binding of the 49 kDa protein to microtubules (Figure 7), and cross-linking of microtubules appeared normal on examination of samples in the electron microscope (not shown).

To study the relationship between isoform composition and phosphorylation, the 49 kDa protein was blotted to nitrocellulose after two-dimensional gel electrophoresis and immunostained. Four spots were detected by antibody to the 49 kDa protein (Figures 8a and 8b) but only those labelled 3 and 4 were revealed by use of the anti-phosphoserine antibody (Figures 8c and 8d). As spot 3 consisted of two isoforms (Figure 2), at least three isoforms may be phosphorylated.

Microfilaments and the 49 kDa protein

Incubation of actin with the 49 kDa protein yielded microfilaments, but the 49 kDa protein failed to bind these polymers and no cross-linking of actin filaments with one another or between actin filaments and microtubules was observed (Figure 9). Interaction of the 49 kDa protein with microtubules occurred in reaction mixtures containing actin (Figure 9a, lanes 9 and 10), an observation verified by electron microscopy. In addition, no obvious correlation was found between the patterns of filamentous actin and the 49 kDa protein on double immunofluorescent staining of *Artemia* larvae with phalloidin and antibody to the 49 kDa protein (not shown).

DISCUSSION

Analysis of taxol-induced microtubule pellets assembled in cell-free extracts of *Artemia* demonstrated that the 49 kDa protein is present at all developmental stages examined, ranging from encysted gastrulae to third-instar larvae. The quantity of 49 kDa protein and tubulin competent to co-assemble increases during development and the ratio of the two proteins declines slightly. The absence of 49 kDa protein in microtubule pellets obtained by a second round of tubulin assembly in *Artemia* extracts demonstrates that quantitative estimates are accurate and not affected by loss of the 49 kDa protein during tubulin assembly. Furthermore, when compared with previous results [15,16], the amounts of tubulin and 49 kDa protein on blots indicate that microtubules prepared by taxol-induced assembly of tubulin in cell-free extracts are not saturated by the 49 kDa protein, a possibility also shown by the decreasing ratio of the two proteins as development proceeds. The partial saturation reflects our earlier immunofluorescent studies [16] in which the 49 kDa protein was localized to specific arrays of microtubules rather than being distributed to all cellular microtubules.

The 49 kDa protein is composed of at least five isoforms with pI values in the range 6.0–6.2. Modification of the IEF gel pH profiles by changing ampholyte ratios did not increase the number of isoforms visible. In two-dimensional gels, the 49 kDa protein, whether purified or in MAP preparations, resolves into four spots, one less than the number of isoforms seen in IEF gels. It is most likely that isoforms 3 and 4, as numbered in IEF gels, merge during electrophoresis in the second dimension. MAP1B, MAP2, tau and MAP4 consist of several isoforms thought to have functional significance, and they vary during development as a result of post-transcriptional processing of mRNA or protein phosphorylation [9,14,32–37]. For example, fibroblasts stably transfected with variant tau isoforms generated by mRNA processing, undergo distinct morphological changes, indicating different roles for isoforms of this MAP in determining cell morphology [36].

The isoform composition of the 49 kDa protein varies little during development of encysted *Artemia* gastrulae to third-instar larvae, a 43 h period, and there is a slight increase in the amount of the protein. These results demonstrate that the 49 kDa protein is synthesized before encystment of the organism and probably throughout development to instar III. Moreover, the function of the 49 kDa protein and its effects on cytoplasmic organization are regulated by means other than differential synthesis of specific isoforms during development, a characteristic contrasting with that exhibited by tau and MAP2. As the major isoforms are unchanged, each may have functional significance, although the range of activities and thus the reasons for the requirement of different isoforms remain uncertain. In some cases, minor isoforms are observed and these may either exist *in vivo* or be generated during sample preparation. The latter possibility is

unlikely because inhibitors of proteolytic enzymes were used and the experiments were repeated at least three times with similar results. Also, minor isoforms appear early in development, a time when proteolytic enzyme activity is low in *Artemia* [38,39].

Are the five isoforms of the 49 kDa protein products of different genes or do they arise by post-transcriptional and post-translational mechanisms? Tau, for example, consists of several isoforms, some of which are formed by phosphorylation [34,35,40–45]. Treatment by alkaline but not acid phosphatase causes a downward mobility shift in the 49 kDa protein on electrophoresis, a modification indicative of dephosphorylation [34,41–47] and suggestive of an effect of phosphorylation on the structure of the 49 kDa protein, as proposed for tau by Larcher et al. [34]. Coomassie staining of gels indicates that there is no proteolytic activity in the alkaline phosphatase, eliminating this as a cause for enhanced mobility of the 49 kDa protein. In addition, blotted 49 kDa protein interacts with an antibody to phosphoserine, a reaction all but eliminated by exposure of blots to alkaline phosphatase. These findings suggest that isoforms of the 49 kDa protein are generated by phosphorylation, a post-translational process. However, only some of the isoforms are phosphorylated at serine, and those that are focus toward the basic end of the IEF gels. Thus phosphorylation of serine contributes to isoform synthesis and explains part of the diversity within the family of 49 kDa proteins. It is possible, however, that each isoform of the 49 kDa protein is the product of a different gene, that phosphorylation at an amino acid residue other than serine contributes to isoform composition, although phosphotyrosine is apparently lacking, or that another post-translational modification affects the number of isoforms. Resolution of this question awaits a more extensive immunological analysis, as well as molecular characterization of the 49 kDa protein by gene cloning and sequencing.

Phosphorylation may inactivate MAPs and cause otherwise intrinsically stable microtubules to become labile [48]. For example, a 170 kDa protein from HeLa cells (pp170) [47] and MAP4 [49,50] lose their ability to bind microtubules and/or their tubulin-assembly-promoting activities when phosphorylated, and phosphorylation of some sites on pp170 is more important for microtubule binding than are others [47]. Phosphorylation of tau has a differential effect in that at some amino acid residues it allows this MAP to bind microtubules whereas at other sites it prevents association [44,50], affecting cell characteristics such as polarity [51]. Phosphorylation of MAP2 at certain sites is also required for binding of this protein to microtubules [41,45]. Our results demonstrate that binding of the 49 kDa protein to microtubules occurs independently of phosphorylation at serine residues, as revealed by the antibody employed in this study.

As MAPs interact with actin [52,53] as well as microtubules, the 49 kDa protein was tested for this activity. Coassembly experiments, carried out under conditions in which the 49 kDa protein binds to microtubules, revealed that this protein does not cross-link or bundle microfilaments. In agreement with this, double immunofluorescent staining of *Artemia* larvae obtained by exposure to phalloidin and an antibody to the 49 kDa protein show that these proteins are in different locations *in vivo*. Also, the 49 kDa protein fails to link microfilaments to microtubules, although the possibility remains that it attaches microtubules to other subcellular components.

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REFERENCES

- Mitchison, T. J. (1992) *Mol. Biol. Cell.* **3**, 1309–1315
- MacRae, T. H. (1992) *Biochem. Cell Biol.* **70**, 835–841
- MacRae, T. H. and Langdon, C. M. (1989) *Biochem. Cell Biol.* **67**, 770–790
- Ludueña, R. F. (1993) *Mol. Biol. Cell* **4**, 445–457
- Brandt, R. and Lee, G. (1993) *J. Biol. Chem.* **268**, 3414–3419
- Goode, B. L. and Feinstein, S. C. (1994) *J. Cell Biol.* **124**, 769–782
- Chapin, S. J. and Bulinski, J. C. (1992) *Cell Motil. Cytoskeleton* **23**, 236–243
- Sharma, N., Kress, Y. and Shafit-Zagardo, B. (1994) *Cell Motil. Cytoskeleton* **27**, 234–247
- Chapin, S. J. and Bulinski, J. C. (1994) *Cell Motil. Cytoskeleton* **27**, 133–149
- Walker, R. A. and Sheetz, M. P. (1993) *Annu. Rev. Biochem.* **62**, 429–451
- Endow, S. A. and Titus, M. A. (1992) *Annu. Rev. Cell Biol.* **8**, 29–66
- Vallee, R. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8769–8772
- Sawin, K. E. and Endow, S. A. (1993) *BioEssays* **15**, 399–407
- MacRae, T. H. (1992) *Biochim. Biophys. Acta* **1160**, 145–155
- Zhang, J. and MacRae, T. H. (1992) *Biochem. Cell Biol.* **70**, 1055–1063
- Zhang, J. and MacRae, T. H. (1994) *J. Biol. Chem.* **269**, 3053–3062
- Masson, D. and Kreis, T. E. (1993) *J. Cell Biol.* **123**, 357–371
- Suprenant, K. A., Dean, K., McKee, J. and Hake, S. (1993) *J. Cell Sci.* **104**, 445–456
- Wang, W., Suprenant, K. and Dentler, W. L. (1993) *J. Biol. Chem.* **268**, 24796–24807
- Michalik, L., Neuville, P., Vanier, M. T. and Launay, J.-F. (1993) *Cell Motil. Cytoskeleton* **25**, 381–390
- Chang-Jie, J. and Sonobe, S. (1993) *J. Cell Sci.* **105**, 891–901
- Schellenbaum, P., Vantard, M. and Lambert, A.-M. (1992) *Biol. Cell* **76**, 359–364
- Hunt, C. and Stebbings, H. (1994) *Cell Motil. Cytoskeleton* **27**, 69–78
- LeClerc, N., Kosik, K. S., Cowan, N., Pienkowski, T. P. and Baas, P. W. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6223–6227
- Kanai, Y., Chen, J. and Hirokawa, N. (1992) *EMBO J.* **11**, 3953–3961
- Campbell, E. J., MacKinlay, S. A. and MacRae, T. H. (1989) *J. Cell Sci.* **93**, 29–39
- Umeiyama, T., Okabe, S., Kanai, Y. and Hirokawa, N. (1993) *J. Cell Biol.* **120**, 451–465
- Weisshaar, B., Doll, T. and Matus, A. (1992) *Development* **116**, 1151–1161
- Langdon, C. M., Rafiee, P. and MacRae, T. H. (1991) *Dev. Biol.* **148**, 138–146
- Langdon, C. M., Freeman, J. A. and MacRae, T. H. (1991) *Dev. Biol.* **148**, 147–155
- Hisanaga, S.-I., Yasugawa, S., Yamakawa, T. et al. (1993) *J. Biochem. (Tokyo)* **113**, 705–709
- Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D. and Crowther, R. A. (1989) *Neuron* **3**, 519–526
- Kosik, K. S., Orecchio, L. D., Bakalis, S. and Neve, R. L. (1989) *Neuron* **2**, 1389–1397
- Larcher, J. C., Boucher, D., Ginzburg, I., Gros, F. and Denoulet, P. (1992) *Dev. Biol.* **154**, 195–204
- Watanabe, A., Hasegawa, M., Suzuki, M. et al. (1993) *J. Biol. Chem.* **268**, 25712–25717
- Lo, M. M. S., Fieles, A. W., Norris, T. E. et al. (1993) *Mol. Brain Res.* **20**, 209–220
- Ulloa, L., Dombradi, V., Diaz-Nido, J. et al. (1993) *FEBS Lett.* **330**, 85–89
- Lu, J. and Warner, A. H. (1991) *Biochem. Cell Biol.* **69**, 96–101
- Pan, B. S., Lan, C. C. and Hung, T. Y. (1991) *Comp. Biochem. Physiol.* **100A**, 725–730
- Greenwood, J. A., Scott, C. W., Spreen, R. C., Caputo, C. B. and Johnson, G. V. W. (1994) *J. Biol. Chem.* **269**, 4373–4380
- Brugg, B. and Matus, A. (1991) *J. Cell Biol.* **114**, 735–743
- Mandelkow, E.-M., Drewes, G., Biernat, J. et al. (1992) *FEBS Lett.* **314**, 315–321
- Goedert, M., Cohen, E. S., Jakes, R. and Cohen, P. (1992) *FEBS Lett.* **312**, 95–99
- Scott, C. W., Spreen, R. C., Herman, J. L. et al. (1993) *J. Biol. Chem.* **268**, 1166–1173
- de Ancos, J. G., Correias, I. and Avila, J. (1993) *J. Biol. Chem.* **268**, 7976–7982
- Takeuchi, M. and Yanagida, M. (1993) *Mol. Biol. Cell* **4**, 247–260
- Rickard, J. E. and Kreis, T. E. (1991) *J. Biol. Chem.* **266**, 17597–17605
- Lieuvain, A., Labbé, J.-C., Dorée, M. and Job, D. (1994) *J. Cell Biol.* **124**, 985–996
- Aizawa, H., Kamijo, M., Ohba, Y. et al. (1991) *Biochem. Biophys. Res. Commun.* **179**, 1620–1626
- Mori, A., Aizawa, H., Saido, T. C. et al. (1991) *Biochemistry* **30**, 9341–9346
- Ferreira, A., Kincaid, R. and Kosik, K. S. (1993) *Mol. Biol. Cell* **4**, 1225–1238
- Fujii, T., Watanabe, M., Ogoma, Y., Kondo, Y. and Arai, T. (1993) *J. Biochem. (Tokyo)* **114**, 827–829
- Correias, I., Padilla, R. and Avila, J. (1990) *Biochem. J.* **269**, 61–64