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A protein related to brain microtubule-associated protein MAP1B is a component of the mammalian centrosome

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SUMMARY

The centrosome is the main microtubule organizing center of mammalian cells. Structurally, it is composed of a pair of centrioles surrounded by a fibro-granular material (the pericentriolar material) from which microtubules are nucleated. However, the nature of centrosomal molecules involved in microtubule nucleation is still obscure. Since brain microtubule-associated proteins (MAPs) lower the critical tubulin concentration required for microtubule nucleation in tubulin solution in vitro, we have examined their possible association with centrosomes. By immunofluorescence, monoclonal and polyclonal antibodies raised against MAP1B stain the centrosome in cultured cells as well as purified centrosomes, whereas antibodies raised against MAP2 give a completely negative reaction. The MAP1B-related antigen is localized to the pericentriolar material as revealed by immunoelectron microscopy. In preparations of purified centrosomes analyzed on polyacrylamide gels, a protein that migrates as brain MAP1B is present. After blotting on nitrocellulose, it is decorated by anti-MAP1B antibodies and the amino acid sequence of proteolytic fragments of this protein is similar to brain MAP1B. Moreover, brain MAP1B and its centrosomal counterpart share the same phosphorylation features and have similar peptide maps.

These data strongly suggest that a protein homologue to MAP1B is present in centrosomes and it is a good candidate for being involved in the nucleating activity of the pericentriolar material.

Key words: centrosome, microtubule-associated protein, MAP1B

INTRODUCTION

The organization of microtubules within most non-neuronal cells appears to depend on the presence of a structure, the centrosome, which has the ability to nucleate the polymerization of tubulin into microtubules (Snyder and McIntosh, 1975; Weisenberg and Rosenfeld, 1975; Frankel, 1976; Osborn and Weber, 1976; Gould and Borisy, 1977; Kuriyama and Borisy, 1981; Mitchison and Kirschner, 1984; Bornens et al., 1987; for review see Bornens et al., 1990; Cande, 1990; Cande and Stearns, 1991; Bornens, 1992; Tucker, 1992).

Electron microscope studies have shown that centrosomes are composed of a pair of centrioles surrounded by an amorphous pericentriolar material (Bornens and Karsenti, 1984), which contains the nucleation sites for microtubules (Gould and Borisy, 1977; Kuriyama and Borisy, 1981; Rieder and Borisy, 1982; for review see Vorobjev and Nadezhdina, 1987; Vandre and Borisy, 1989; Bornens, 1992). Little is known about the organization of the molecular components of centrosomes. Several proteins have been localized to the centrosome by immunocytochemistry and although the presence of a nucleic acid (either DNA or RNA) has been suggested (for reviews see Bornens and Karsenti, 1984; Vorobjev and Nadezhdina, 1987) it still remains controversial (Hall et al., 1989; Klotz et al., 1990; Johnson and Dutcher, 1991; for review see Johnson and Rosenbaum, 1991).

A first step in the characterization of centrosomal components is the isolation of functional centrosomes (Mitchison and Kirschner, 1984; Bornens et al., 1987; Komesli et al., 1989). These procedures are mainly based on the fractionation through sucrose gradients of cell homogenates obtained after hypotonic lysis of cultured cells previously incubated with cytoskeletal-disrupting drugs (Maro and Bornens, 1980; Bornens et al., 1987). The above mentioned procedures have been useful for functional studies, but biochemical studies have been hampered by the low yields of centrosome purifications and the large and variable amounts of different proteins present in the centrosomal preparations. To overcome these problems, immunological probes of known proteins have been used to correlate biochemical analyses of centrosome preparations with immunocytochemical studies (Gosti-Testu et al., 1986, 1987; Bornens et al., 1987; Balczon and West, 1991; Moudjou et al., 1991; Stearns et al., 1991; Zheng et al., 1991; Chevrier et al., 1992; Joshi et al., 1992; Sellitto et al., 1992; Tucker et al., 1992; Rothbarth et al., 1993; Keryer et al., 1993; for review Kuriyama, 1992; Kalt and Schliwa, 1993). The centrosomal

components responsible for the nucleation of the assembly of microtubules have not been identified yet. Interestingly, Moudjou et al. (1991), have found a 62-64 kDa centrosomal protein that could be involved in the nucleation process. Recently the presence of a new member of the tubulin family, the γ-tubulin, has been described as being localized at the microtubule organizing centers of eukaryotic cells (Zheng et al., 1991; Stearns et al., 1991; for review, see Oakley, 1992). It seems to be required for microtubule polymerization initiation in vivo (Joshi et al., 1992). Probably, γ-tubulin will act as a template for the initiation of the polymerization of tubulin at the microtubule organizing centers. Its function in stimulating nucleation of polymer growth is still unclear (Joshi et al., 1992; Oakley, 1992).

As microtubule-associated proteins (MAPs) present in neuronal cells favor the in vitro nucleation of polymer assembly (Olmsted, 1986), we have searched for proteins related to brain MAPs in centrosomes from non-neuronal cells by combining immunocytochemical with biochemical analyses of centrosomal preparations. In the present study we show by immunological analysis, phosphorylation characteristics, peptide mapping, and partial sequence analysis, that a protein related to brain microtubule-associated protein MAP1B (also referred to as MAP5, MAP1.2 or MAP1X) is indeed present in isolated mammalian centrosomes and localized to the pericentriolar material.

MATERIALS AND METHODS

Cell culture

Human lymphoblastic KE37 (Mayer et al., 1982) cells were cultured in RPMI1640 medium containing 10% fetal calf serum at 37°C and 5% CO_2 in air. The *Xenopus laevis* epithelial cell line XL177 (Miller and Daniel, 1977), was cultured in 55% Leibovitz L15 medium (Gibco, UK), 15% fetal calf serum, at 25°C. Mouse Ehrlich-Lettre (ATCC CCL77) cells were injected intraperitoneally into three-month-old Balb/c mice. Nine days after injection, ascitic fluid was extracted and the cells isolated by centrifugation at 1500 ***g***.

Antibodies

Monoclonal antibodies (356 and 357) against α- and β-tubulin subunits were purchased from Amersham (UK). The characterization of the monospecific rabbit antibodies against brain MAP2 used in this work has been done previously (Hernandez et al., 1989). Monoclonal antibody against MAP2 (AP-20) was purchased from Boehringer Mannheim. Monoclonal antibody against MAP1B 125 was already described (Ulloa et al., 1993a,b), monoclonal antibody 150 against phosphorylated MAP1B was a kind gift from Dr Moya (Mansfield et al., 1992). Polyclonal antiserum against MAP1B, 81 has already been described (Diaz-Nido and Avila, 1989a). The antibodies from the autoimmune human serum that specifically recognizes a centrosomal protein will be described (Tournier et al., 1991; Domínguez et al., unpublished). We also used the 5051 human auto antibody from Kirschner's lab (Calarco-Gillam et al., 1983) and the monoclonal antibody from Bornens (CTR453; Bailly et al., 1989).

Isolation of centrosomes

The protocol of Bornens et al. (1987) was followed with slight modifications: (a) step 5 was repeated three times; (b) the supernatant of the lysis (step 6) was overlaid on a 50% sucrose (w/w) cushion prepared in 10 mM K-PIPES, pH 7.2, 1 mM EDTA, 0.1% 2-mercaptoethanol and 0.1% Triton X-100, and centrifuged at 40,000 ***g*** for 20 minutes. The interface between the lysate and the 50% sucrose cushion was collected and overlaid on the discontinuous sucrose gradient system (Bornens et al., 1987).

Immunofluorescence microscopy

Centrosomes were sedimented onto coverslips as previously reported (Evans et al., 1985), fixed with −20°C methanol and washed with phosphate buffered saline buffer (PBS). The coverslips were incubated for 30 minutes with the antibodies and after three 10 minute washes with PBS, they were further incubated with fluorescein- or rhodamine-conjugated goat anti-mouse, goat anti-human or goat anti-rabbit immunoglobulins (Tago, CA; Jackson Immunoresearch Labs, PA; Cappel, PA). To check the DNA contamination of the centrosomal purification steps, the dye Hoechst No. 33258 (Sigma, St Louis, MO) was added to the secondary antibody solution at a final concentration of 10 μg/ml. The coverslips were mounted with Mowiol R 40-88 (Aldrich Chemie) and examined using a Zeiss epifluorescence microscope.

Immunoelectron microscopy

The isolated centrosomes were sedimented onto 12 mm round coverslips, fixed in 0.25% glutaraldehyde in PBS and incubated twice with 0.1% (w/v) sodium borohydride (Sigma, St Louis, MO) in the same buffer. After a 10 minute incubation in fetal calf serum (10% in PBS), the preparation was incubated with the anti-MAP1B (125) antibody (1/5 dilution from hybridoma-conditioned culture medium) for 15 minutes at room temperature followed by rabbit anti-mouse (1/150 dilution; Jackson Immunoresearch Labs, PA) for 30 minutes at room temperature and Protein A coupled to 9 nm gold particles (1/75 dilution; Janssen Pharmaceutica, Belgium) for 30 minutes at room temperature. The preparation was postfixed with 1% glutaraldehyde and 2% osmium tetroxide (Merck, Germany). The centrosomes were finally stained with aqueous uranyl acetate and embedded in Epon. Sections parallel to the coverslips were observed in the electron microscope (model 400, Philips) after contrasting with uranyl acetate and lead citrate.

A negative control was made using the monoclonal antibody against MAP2, AP-20, (1/200 dilution).

Protein preparation and analysis

Microtubule proteins from mouse brain were prepared through temperature-dependent cycles of assembly-disassembly as indicated by Shelanski et al. (1973) with the modifications of Karr et al. (1979). Tubulin was purified as described by Weingarten et al. (1975). Newborn mouse brain MAPs were purified as described by Vallee et al. (1986). Protein composition from microtubule or centrosome fractions was analyzed by gel electrophoresis according to the procedure of Laemmli (1970). Gels were either stained with Coomassie Blue as indicated by Fairbanks et al. (1981) or with silver as indicated by Harlow and Lane (1988). Western blotting analyses were carried out by transferring the proteins previously fractionated by gel electrophoresis to nitrocellulose membranes, according to Towbin et al. (1979). A peroxidase-conjugated secondary antibody was used and the peroxidase reaction was developed using 3,3′-diaminobenzidine (Sigma, St Louis, MO) and hydrogen peroxide as substrates. Partial amino acid sequence of the peptides from centrosomal proteins was carried out after transferring the peptides previously fractionated by gel electrophoresis to Immobilon sheets (Millipore, MA) as indicated by Vandekerckhove et al. (1985). The peptides on Immobilon were then subjected to automated Edman degradation cycles in a gas-phase sequenator (Applied Biosystems) equipped with an on line phenylthiohydantoin amino acid analyzer (model 120A).

Peptide mapping

Peptide mapping with *Staphylococcus aureus* V8 protease (Sigma, St Louis, MO) was done according to the method of Cleveland et al. (1977).

NTCB (2-nitro-5-thiocyanobenzoic acid) peptide mapping was done as described by Correas et al. (1992) with slight modifications: the protein bands were stained by the imidazole-zinc reverse staining method as described by Ortiz et al. (1992). NTCB was from Sigma (St Louis, MO).

Phosphorylation assays

Endogenous phosphorylation of centrosomal and microtubule preparations was carried out in 0.1 M MES, pH 6.4, supplemented with 2 mM EGTA, 6 mM $MgCl_2$ and 10 μM [γ-^{32}P]ATP (Amersham, UK) either in the presence or the absence of 10 μg/ml poly-L-lysine (an activator of casein kinase II; Hathaway and Traugh, 1982) plus 10 μM cAMP-dependent protein kinase inhibitor (Sigma, St Louis, MO), or 1 μM heparin (an inhibitor of casein kinase II; Hathaway and Traugh, 1982). The mixture was incubated for 20 minutes at 37°C and the reaction was stopped with SDS-sample buffer (Laemmli, 1970). Samples were analyzed by electrophoresis, and phosphorylated proteins were subjected to V8 protease peptide mapping, as described above.

Microtubule-binding assay of the MAP1B-related protein

Purified centrosomes (20 μg of protein) were extracted with 2 M KI as described by Klotz et al. (1990). The extracted material was dialyzed and concentrated in a negative pressure protein dialysis concentrator (Bio-Molecular Dynamics, OR). The dialysis was made against 0.1 M MES, pH 6.4, 6 mM $MgCl_2$, 2 mM EGTA. After dialysis the protein concentration was measured (Smith et al., 1985) and [γ-^{32}P]ATP was added to a concentration of 1 μM in the presence of 1 mM PMSF and 10 μg/ml poly-L-lysine. The whole mixture was incubated for 20 minutes at 37°C. The phosphorylation reaction was stopped with NaF and cold ATP added to a final concentration of 50 mM and 1 mM, respectively. Just before copolymerization with microtubules, this solution was centrifuged at 100,000 ***g*** for 10 minutes at 4°C in a Beckman TL100 ultracentrifuge (TL100.1 rotor), to remove aggregates.

Phosphocellulose-purified tubulin was polymerized in 0.1 M MES, pH 6.4, 0.5 mM $MgCl_2$, 2 mM EGTA, 20 mM NaF, 1 mM ATP, 1 mM GTP for 20 minutes at 37°C. The polymerized microtubules were mixed with the KI-extracted centrosomal proteins and incubated at 37°C for 15 minutes.

The mixture was centrifuged at 100,000 ***g***, for 10 minutes at 25°C in a Beckman TL100 ultracentrifuge (TL100.3 rotor). The pellet and supernatant were boiled in electrophoresis sample buffer (Laemmli, 1970), for 5 minutes. The proteins were analyzed by electrophoresis in a 10% polyacrylamide gel.

Assay of microtubule nucleating activity of centrosomes

Phosphocellulose purified tubulin was mixed (at a final concentration of 15 μM) with isolated centrosomes in the conditions described by Mitchison and Kirschner (1984). The incubation buffer was 80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP. The microtubule regrowth from centrosomes was allowed for 10 minutes at 37°C and the reaction stopped by fixation with 0.7% glutaraldehyde. Afterwards, tubulin immunofluorescence was performed as previously described (Buendia et al., 1992). We assayed three different conditions: absence of added antibodies, 20 minutes pre-incubation with 125 hybridoma cell culture supernatant at a final dilution of 1/10 at room temperature, and pre-incubation with a hybridoma (IgM) cell culture supernatant against a protein fraction from Golgi apparatus.

RESULTS

An antigen related to brain MAP1B protein is localized to the centrosome in cultured cells

As shown in Fig. 1, in KE37 human lymphocytes, mouse Ehrlich-Lettre cells and frog cells, a MAP1B-related antigen is present on a cytoplasmic structure corresponding to the centrosome and to some extent in the nuclei. This was determined by double immunofluorescence using different anti-centrosome antibodies, two human scleroderma sera, one of which is the 5051 serum described by Kirschner's group, and the other is a serum we have recently characterized (our unpublished observations; also see Tournier et al., 1991; Calarco-Gillam et al., 1983). We also used one monoclonal antibody raised against isolated centrosomes (CTR453; from M. Bornens group, Bailly et al., 1989) in conjunction with polyclonal anti-MAP1B antibodies. In Fig. 1b,d a monoclonal antibody to MAP1B (125) was used and in Fig. 1e,g a polyclonal antibody raised against MAP1B was used. Note the strong and very clean staining of a cytoplasmic spot located close to the nucleus that corresponds to the centrosome as well as a dotted staining in the nucleus. The identity of the cytoplasmic dot as the centrosome is further confirmed by its position centered at the origin of the microtubule network (Fig. 1a,c). We have used three different anti-MAP1B antibodies (81, 125 and 150) that all gave a similar staining in all cells. A similar staining pattern has also been described in other cell lines (rat, mouse and chinese hamster; Diaz-Nido and Avila, 1989a).

Localization of the protein immunologically related to brain MAP1B in isolated centrosomes

We have used centrosomes prepared as described by Bornens et al. (1987), to examine the potential physical association of a MAP1B-like protein with these structures. The quality of centrosomal preparations was monitored by immunofluorescence using double immunostaining with anti-tubulin and anti-centrosomal antibodies. The DNA-binding dye Hoechst 33258 (Weisblum and Haenssler, 1974) was systematically included in the assay to test for potential contamination by DNA. The final centrosome preparations were virtually devoid of contaminating DNA, and many centrosomes appeared as pairs of dots as previously reported (data not shown; see Mitchison and Kirschner, 1984; Bornens et al., 1987). The potential presence of MAP1B-like proteins was examined by double immunofluorescence using our human centrosomal auto-antibody and the monoclonal 125 raised against MAP1B, or the monoclonal antibody against MAP2 as a negative control (Fig. 2). The anti-MAP1B but not the anti-MAP2 antibody stained the centrosomes strongly. The colocalisation of the anti-MAP1B staining with dots stained by the anti-centrosome antibodies was pretty good, suggesting that the MAP1B-related protein was indeed present in the isolated centrosomes. The dots were often in pairs as is the case for isolated centrosomes. The proportion of paired centrioles in isolated centrosomes depends on the quality of the preparation and the buffer used to lyse the cells. In our preparations it ranges from 30% to 50% as determined by electron microscopy on negatively stained samples (D. Chrétien, unpublished data). In this experiment, we found about 30% of centrosomes as pairs of dots using the anti-centrosome antibody, all of which were also stained by the anti-MAP1B antibody. This co-localization was confirmed by immunogold electron microscopy. As shown in Fig. 3, the staining was found associated with the pericentriolar material. The lack of staining inside the pericentriolar material does not mean that the antigen is only present at the periphery since there might be a problem of penetration. No pericentriolar

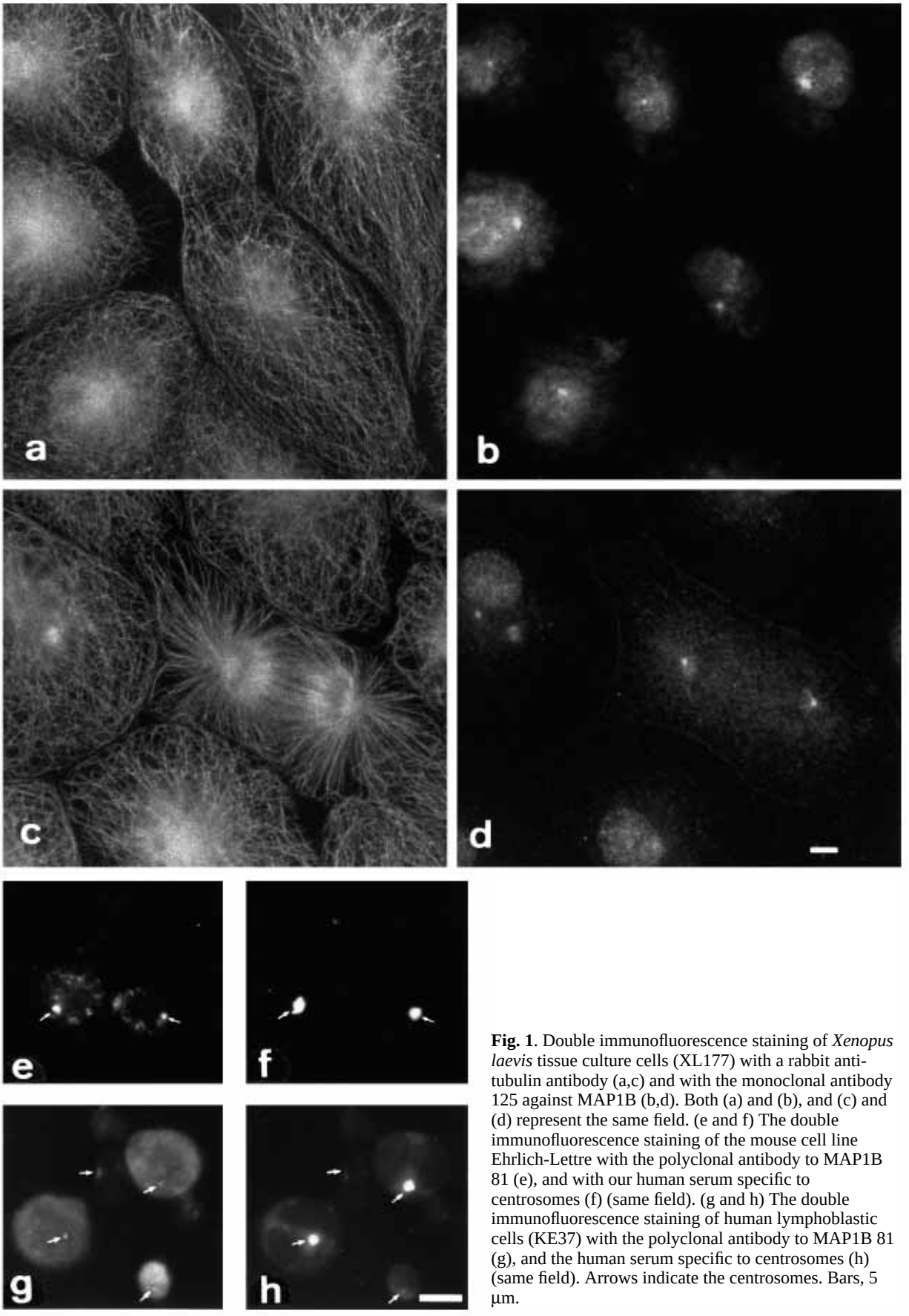


Fig. 1. Double immunofluorescence staining of *Xenopus laevis* tissue culture cells (XL177) with a rabbit anti-tubulin antibody (a,c) and with the monoclonal antibody 125 against MAP1B (b,d). Both (a) and (b), and (c) and (d) represent the same field. (e and f) The double immunofluorescence staining of the mouse cell line Ehrlich-Lettre with the polyclonal antibody to MAP1B 81 (e), and with our human serum specific to centrosomes (f) (same field). (g and h) The double immunofluorescence staining of human lymphoblastic cells (KE37) with the polyclonal antibody to MAP1B 81 (g), and the human serum specific to centrosomes (h) (same field). Arrows indicate the centrosomes. Bars, 5 μm.

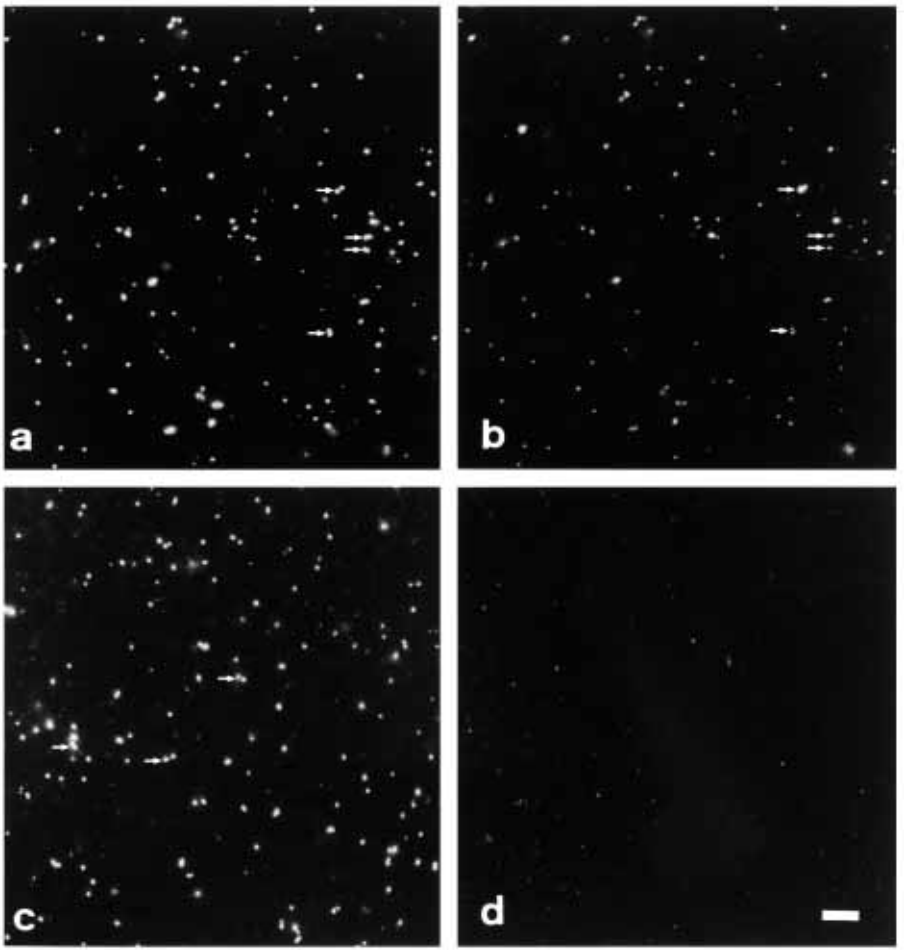


Fig. 2. Double immunofluorescence staining of purified centrosomes labeled with antibodies to: (a,c) centrosomes (human auto-immune serum); (b) MAP1B (monoclonal 125); and (d) a MAP2 monoclonal. Arrows indicate some centrosomal doublets. Bar, 10 μm.

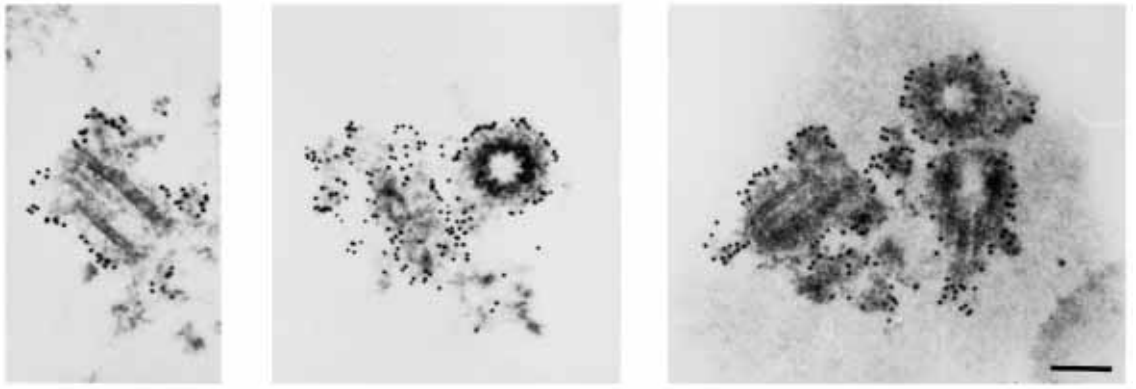


Fig. 3. Immunoelectron microscopy of centrosomes incubated with the monoclonal antibody against MAP1B (125) and revealed by a rabbit anti-mouse immunoglobulins and Protein A coupled to 9 nm gold particles. Bar, 0.2 μm.

staining was found with anti-MAP2 antibody that was used as negative control in the experiment (not shown).

In order to characterize the polypeptides present in the centrosomes, 2×10^9 centrosomes containing about 50 μg of protein were subjected to gel electrophoresis. Fig. 4 shows the polypeptide pattern of a mouse centrosomal preparation analyzed on a 5% acrylamide gel in urea. In addition to one major protein doublet migrating with an electrophoretic mobility of about 230 kDa, the centrosome preparation contained one electrophoretic band co-migrating with brain MAP1B (Fig. 4A,B). This latter protein band reacted with different anti-MAP1B antibodies by western blotting analysis. Fig. 4C shows the result obtained with the polyclonal antibody (81) against MAP1B (see arrow). The same result was obtained with a human centrosome preparation. Looking closely at the western blot (Fig. 4C) the MAP1B immunoreactivity can be seen in the form of a doublet. This could reflect the presence in this centrosomal protein of two different isoforms that arise by its differential phosphorylation features (Riederer et al., 1990; Ulloa et al., 1993a). Fig. 4, section 2, shows the electrophoretic analysis of purified centrosomes (50 μg) on a 10-20% linear gradient polyacrylamide gel after staining with Coomassie Blue. The absence of any detectable protein of electrophoretic mobility below 45 kDa, and a high abundance of proteins in the range of 45-68 kDa is clear.

Several cytoskeletal proteins like spectrin and myosin have

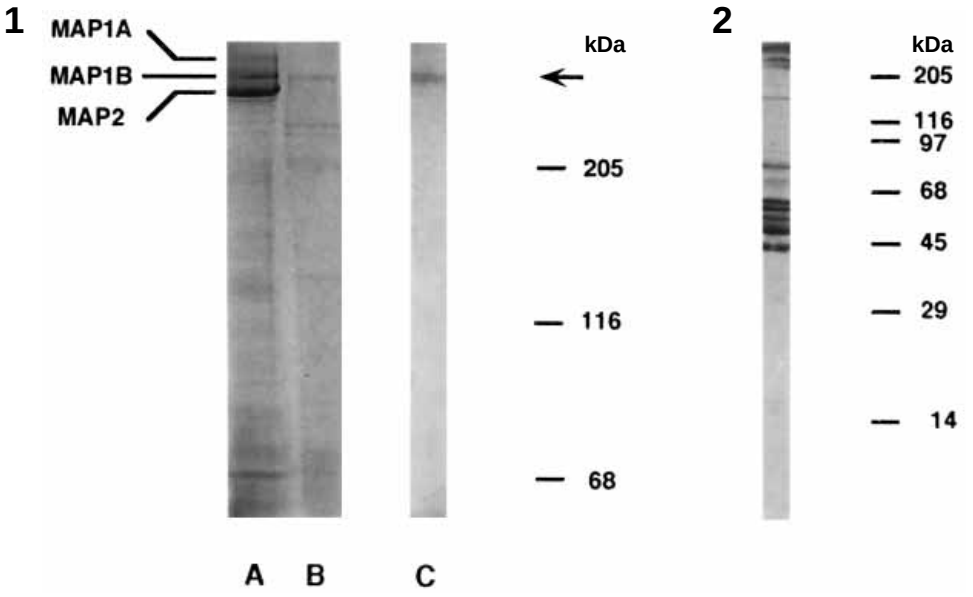


Fig. 4. SDS-polyacrylamide gel electrophoresis and immunoblotting analysis of centrosomal proteins from mouse Ehrlich-Lettre cells. (1) The proteins were ran on a 5% acrylamide gel containing 4 M urea. Lane A, mouse brain microtubule proteins; lane B, centrosomal proteins (Coomassie staining); lane C, centrosomal proteins transferred to nitrocellulose and stained with rabbit polyclonal antibody to MAP1B 81. A centrosomal protein with similar mobility to mouse brain MAP1B is stained by this antibody (see arrow). The other antibodies against MAP1B gave similar results. (2) Centrosomal proteins (Coomassie staining) analyzed on a 10-20% linear gradient of polyacrylamide. Sizes of marker proteins (in kDa) are indicated at the right margin.

been found in centrosomal preparations and are suspected to be mere contaminants that could either sediment like centrosomes on sucrose gradients or become adsorbed non-specifically to them during lysis of the cells in low ionic strength buffer (Komesli et al., 1989). This does not seem to be the case for MAP1B, since it can be detected by immunofluorescence at the centrosome of cultured cells. To strengthen the case, we first compared the electrophoretic pattern of the final centrosome preparation with the protein composition of the lysed cells (Fig. 5). The band co-migrating with brain MAP1B and stained by the anti-MAP1B antibodies (Figs 4 and 5) was enriched in the final centrosome preparation (Fig. 5A), whereas it was not visible in the cell lysate. The enrichment of this band was concomitant with an increment in the anti-MAP1B immunoreactivity (Fig. 5B). At the moment we do not know if this 325 kDa band actually corresponds to a single protein. We have also analyzed by dot blot the distribution of the MAP1B-related antigen in the sucrose gradient used to purify centrosomes. MAP1B-related protein co-migrated exactly with mouse Ehrlich-Lettre cell centrosomes (not shown). The same result was obtained with centrosomes isolated from human lymphoblastic cells.

Taken together these results indicate that MAP1B copurifies with, and becomes enriched in, centrosomes, arguing against it being a mere contaminant of the centrosome preparation.

Characterization of the centrosomal MAP1B-related protein

In order to check for structural similarities between the centrosomal MAP1B-related protein and brain MAP1B, it was of interest to have some information about the amino acid sequence of the centrosomal protein. The centrosomal MAP1B-related protein turned out to have a blocked amino-terminal residue, just like brain MAP1B. Interestingly, in some centrosome preparations, low molecular mass polypeptides were recognized by anti-MAP1B antibodies, suggesting that they were degradation products of the complete molecule. We took advantage of this feature and carried out an experiment in which proteolytic degradation was enhanced purposely by

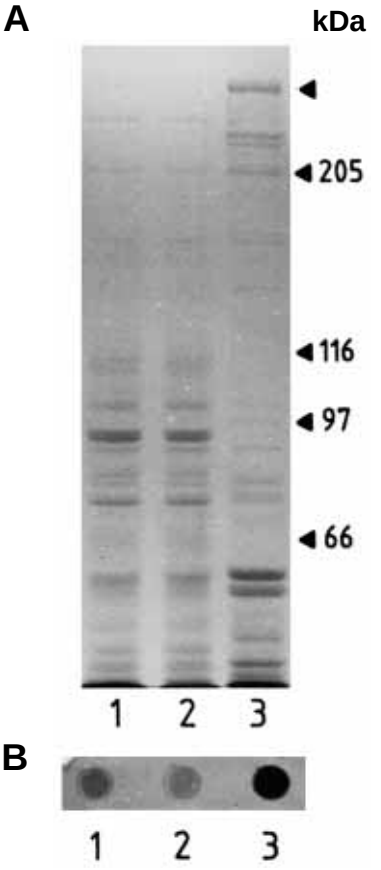


Fig. 5. The MAP1B-related protein is enriched in purified centrosomes. In lysed cells (A, lane 1) or in centrosomes recovered after the concentration step (A, lane 2), there is no visible protein migrating as MAP1B whereas in the purified centrosomes (A, lane 3), a 325 kDa band appears (arrowhead). Sizes (in kDa) of marker proteins are indicated to the right margin. Dot blot analysis on 20 μg of protein from each fraction (B) shows a dramatic increase in immunoreactivity for the polyclonal antibody to MAP1B (81) in the purified centrosome fraction (1, 2 and 3 correspond, respectively, to lysed cells, concentration step and purified centrosomes).

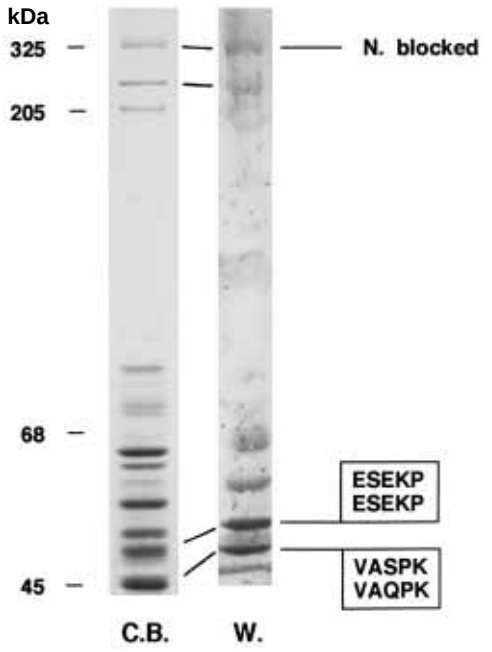


Fig. 6. Amino acid sequence of two low molecular mass centrosomal peptides recognized by the anti-MAP1B antibody. Centrosomal proteins were separated on a 6.5% polyacrylamide gel and stained with Coomassie Blue (C.B.) or transferred to nitrocellulose and stained with the anti-MAP1B antibody 81 (W.). Proteolytic degradation was enhanced by incubation of the centrosomal proteins for 120 minutes at 25°C. Several peptides (probably degradation products of the high molecular mass protein) reacted with the antibody. Those found in higher amounts were transferred to Immobilon sheets and their amino terminal residues sequenced as indicated in Material and Methods. Insets show the sequence of the centrosomal peptides corresponding to the sequence of brain MAP1B (Noble et al., 1989). Sizes (in kDa) of marker proteins are indicated at the left margin.

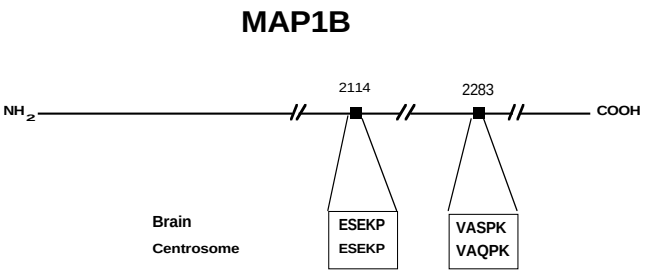
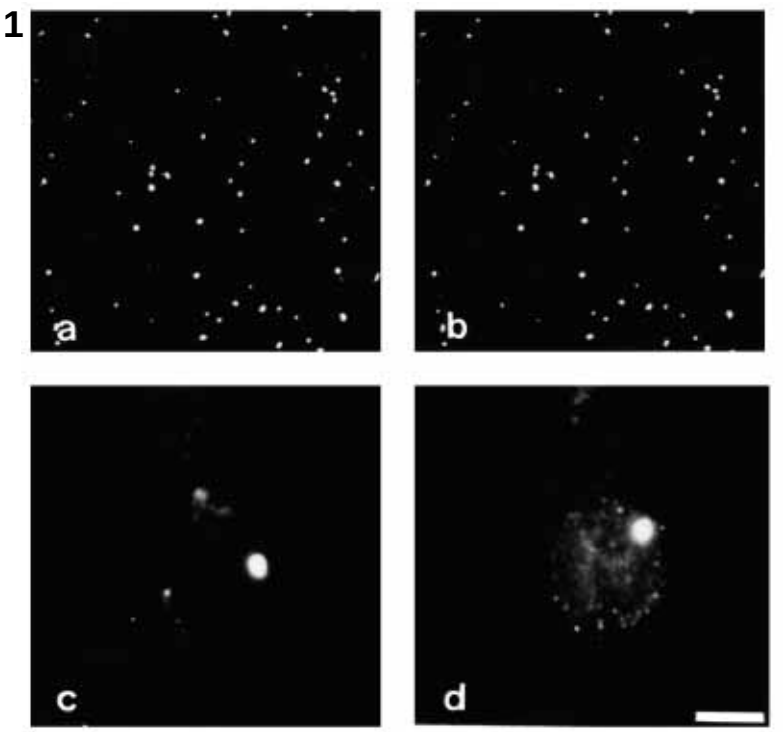


Fig. 7. Possible alignment of the centrosomal low molecular mass peptides with the amino acid sequence of mouse brain MAP1B.

incubation of a centrosomal preparation for 120 minutes at 25°C. Two major peptides of 48 and 45 kDa were generated from MAP1B after this incubation as they react with the anti-MAP1B polyclonal antibody 81 (Fig. 6). Both 48 kDa and 45 kDa peptides were sequenced by Edman degradation. A major (almost unique) sequence for the amino-terminal regions of 45 and 48 kDa peptides strongly supports the presence of single polypeptides of these molecular masses after the mild proteolysis conditions assayed. The sequence for the first five amino acid residues of the 48 kDa centrosomal peptide was identical to that found in residues 2114 to 2118 of mouse brain MAP1B (Noble et al., 1989). The other centrosomal peptide (45 kDa) had an N-terminal sequence similar to that of residues 2283 to 2287 of mouse brain MAP1B with a glutamine where brain MAP1B has a serine (position 2285; Noble et al., 1989). The putative localization of these peptides in the brain MAP1B molecule is indicated in Fig. 7.

Brain MAP1B protein is phosphorylated by a casein kinase II-like enzyme (Diaz-Nido et al., 1988) and probably by proline-dependent protein kinases (Ulloa et al., 1993a). Casein kinase II has a cytoplasmic distribution that could involve its location, at least in some stages of the cell cycle, in the centrosome (Serrano et al., 1989; and Fig. 9 of Yu et al., 1991, suggests its presence in the mitotic spindle); the same affirmation is applicable to the $p34^{cdc2}$ protein kinase (Bailly et al., 1989). As shown in Fig. 8 (panel 1), isolated centrosomes as well as centrosomes in whole cells were stained by the monoclonal antibody 150, which recognizes a phosphorylated epitope on brain MAP1B (Fig. 8, panel 2; and Ulloa et al., 1993a). This suggested that the centrosomal MAP1B was indeed phosphorylated in vivo. We therefore incubated isolated centrosomes with [γ-^{32}P]ATP. As shown in Fig. 9A, a 325 kDa protein became phosphorylated. This phosphorylation was increased in the presence of poly-L-lysine, an activator of casein kinase II and reduced in the presence of heparin, an inhibitor of this enzyme (Fig. 9B; Hathaway and Traugh, 1982). This suggested that a casein kinase II-related enzyme was present in the centrosome preparations and that it may phosphorylate a centrosomal protein having a similar molecular mass to that of brain MAP1B.

In order to identify further this protein as a MAP1B-related polypeptide, we analyzed the peptide maps obtained after two different proteolytic cleavages of the 325 kDa protein present in centrosome preparations. First, the 325 kDa centrosomal band was excised and cleaved using the cysteine cleaving agent NTCB. Brain MAP1B and myosin (as negative control), were processed in the same way. Both brain MAP1B and the centrosomal protein showed similar but not identical NTCB-peptide maps (lanes 1 and 2, Fig. 9C), that were clearly different from the one obtained with muscle myosin (lane 3, Fig. 9C). Most of the peptides originating from the centrosomal protein were present in brain MAP1B, but this latter protein gave rise to additional polypeptides. This could be due to the presence of proteins and/or proteolytic fragments from higher molecular mass proteins (for example, MAP1A) comigrating with brain MAP1B in the first gel or to the existence of some structural differences between brain MAP1B and its centrosomal counterpart (see above). To further confirm the identity of the phosphorylated protein shown in Fig. 9A, the ^{32}P-labeled band was exposed to V8 protease digestion and the products compared to those generated from brain MAP1B and MAP2 phosphorylated by casein kinase II. The phosphopeptide maps of the centrosomal protein and brain MAP1B turned out to be similar and clearly different from that of brain MAP2 (Fig. 9D). Again, the peptide map of the brain protein was slightly more complex than that of the centrosomal protein and the explanation put forward for the NTCB cleavage is also probably valid here. This could reflect distinctive phosphorylation features of both proteins or the presence of proteolytic fragments from MAP1A with the same electrophoretic mobility as brain MAP1B.



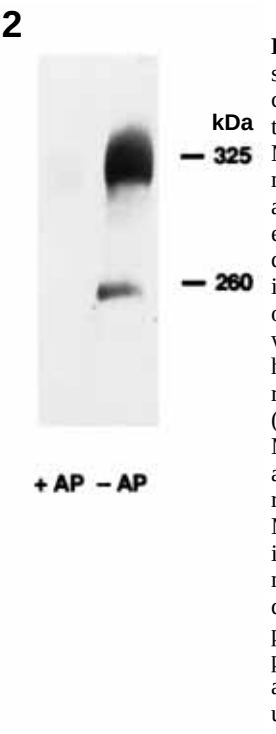


Fig. 8. (1) Immunofluorescence staining of isolated centrosomes incubated (a) with the polyclonal antibody to MAP1B 81, and (b) with the monoclonal antibody (150) against a phosphorylated epitope on this protein. (c and d) A double immunofluorescence staining of mouse Ehrlich-Lettre cells with monoclonal 150 and the human anti-centrosome serum, respectively. Bar, 5 μm. (2) Western blotting of brain MAPs incubated or not with alkaline phosphatase and resolved on a 5% acrylamide, 4 M urea electrophoresis gel. The immunoreactivity with the monoclonal antibody 150 decreases upon removal of phosphate groups from the protein. +AP, treated with alkaline phosphatase; −AP, untreated.

In summary, the 325 kDa protein present in isolated centrosomes shares with the brain MAP1B its molecular mass, antigenic cross reactivity, partial microsequence identity, peptide maps and some phosphorylation characteristics. Although definitive identification will require cloning of this protein, this is a pretty strong case.

Microtubule nucleation of centrosomes in the presence of anti-MAP1B antibodies

To look for a possible functional role of the centrosomal MAP1B-related protein in microtubule nucleation the centrosome-nucleating activity in the presence or absence of the purified monoclonal antibody against MAP1B 125 was tested. The number of microtubules per centrosome in the control (no antibody added) was 26±18, whereas in the presence of the antibody against Golgi proteins it was 26±20 and that found in the presence of antibody 125 against MAP1B was 18±9. A total of 50 centrosomes were counted. This result indicates a slight decrease, probably not significant, in the number of microtubules per centrosome when anti-MAP1B antibody was present.

DISCUSSION

Immunofluorescence staining of mammalian cell centrosomes by antibodies raised against brain microtubule-associated protein MAP1 had been reported by several authors (Sherline and Mascardo, 1982; Sato et al., 1983; De Mey et al., 1984; Bonifacino et al., 1985; Vallee et al., 1986; Diaz-Nido and Avila, 1989a). However, these immunocytochemical studies did not provide evidence for a tight interaction between the MAP1B-related antigen and centrosomes. Moreover, immunological cross reaction is not sufficient to establish that the centrosomal antigen is closely related to the known brain MAP1B protein. In the present work, we have shown that a protein related to brain MAP1B is indeed present in purified mammalian centrosomes. The relationship between the centrosomal protein and brain MAP1B has been established by several criteria: (i) specific antibodies directed against MAP1B stain cultured cells and purified centrosomes by immunofluorescence and recognize a protein with a similar molecular mass to brain MAP1B on immunoblots of centrosomal proteins; (ii) a partial amino acid sequence of degradation products of the centrosomal protein is very similar to that of a region of brain MAP1B (Noble et al., 1989); (iii) both proteins can be phosphorylated by a casein kinase II-like enzyme giving similar phosphopeptides that are different from those obtained from MAP2; (iv) the peptidic maps of both proteins are similar. Finally, preliminary results indicate that the centrosomal MAP1B-related protein binds to microtubules. This was done by extraction of centrosomal proteins using KI as described by Klotz et al. (1990) followed by dialysis and binding to microtubules after in vitro phosphorylation of the protein (see Materials and Methods). In the extracted fraction, the only protein that is phosphorylated and binds to microtubules has a molecular mass of 325 kDa, which strongly suggests that it is the MAP1B-related protein we have characterized. However, because of the difficulties encountered when preparing this fraction, we have not been able to identify it positively in the same experiments.

We have localized the centrosomal MAP1B-related polypeptide at the periphery of the pericentriolar material by immunoelectron microscopy. Obviously, this does not exclude its presence inside the pericentriolar material. In any case, such a localization is compatible with a role for this MAP in the nucleation of microtubules by centrosomes. Indeed, brain MAP1B has been shown to have microtubule promoting activity in vitro (Riederer et al., 1986; Diaz-Nido and Avila, 1989b). However, the addition of a purified antibody against

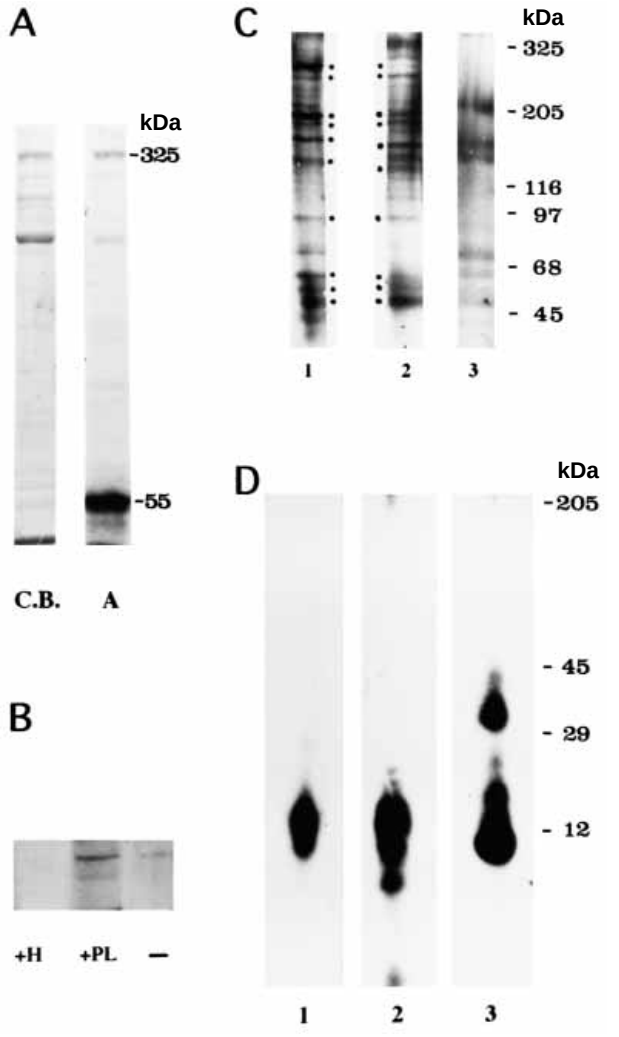


Fig. 9. Autophosphorylation of centrosomal proteins. (A) Purified centrosomes were incubated with 10 μM [γ-^{32}P]ATP under the conditions indicated in Materials and Methods in the presence of 10 μg/ml of poly-L-lysine plus 10 μM cAMP-dependent protein kinase inhibitor. Lane C.B., Coomassie staining of the preparation used in this experiment; lane A, ^{32}P incorporation into the proteins (autoradiography of gel C.B.). The percentage of acrylamide of the electrophoresis gel was 6.5% plus 2 M urea. (B) Incorporation of ^{32}P in the 325 kDa protein is inhibited by heparin (+H), and increased by poly-L-lysine (+PL) compared to the incorporation observed without either of these compounds (–). (C) Comparative peptide mapping obtained by NTCB proteolysis (as described in Material and Methods) of the centrosomal 325 kDa protein (lane 1); mouse brain MAP1B (lane 2); and myosin (lane 3). Proteins on the 5-15% acrylamide electrophoresis gradient were detected after silver staining. Black dots indicate the coincident peptides between brain and centrosomal MAP1B (D) Phosphopeptides from mouse centrosomal MAP1B (lane 1), mouse brain MAP1B (lane 2) and MAP2 (lane 3). Phosphopeptides were generated by V8 proteolysis of phosphoproteins cut out from acrylamide gels (Cleveland et al., 1977), separated on 7.5-20% gradient acrylamide gels and identified by autoradiography. Sizes (in kDa) of marker proteins are indicated at the right margins.

MAP1B (125), does not clearly inhibit the microtubule nucleating activity of purified centrosomes. There are two alternative explanations for this result; the first one is that there could be a cooperative effect between MAP1B and other microtubule nucleating molecules such as γ-tubulin; the second could be that the region recognized by antibody 125 on the MAP1B molecule is not involved in this function. There are several indirect data compatible with a function for MAP1B-related protein in nucleation. For example, preliminary experiments have shown that after centrosomal inactivation by urea treatment (Klotz et al., 1990), MAP1B protein is released. Moreover, in tubulin solutions, MAPs (such as MAP1B) have been described to have the property of stabilizing microtubules and they also promote microtubule nucleation by lowering the critical concentration required for polymerization. Therefore, in vivo, a MAP that is soluble in a given cell type could favor spontaneous, random nucleation and stabilize microtubules, whereas in an other cell type it could be targeted by linker molecules to insoluble structures, like centrioles or pericentriolar material, where it would induce local microtubule nucleation. MAP1B could belong to this class of MAP. A second group of MAPs would include the MAP2/tau/MAP4 family. It has previously been shown that both MAP2 and tau can increase the number of microtubules nucleated by isolated centrosomes (Bré et al., 1987) and these MAPs were postulated to increase the nucleating activity of centrosomes by reducing microtubule instability rather than through binding directly to the centrosomes. This function could be achieved in non-neuronal cells by MAP2-tau-related proteins like MAP4. Additionally MAP1B, or immunologically related proteins, have been described in all the tissues so far tested. The expression of these proteins is developmentally regulated in brain, though in the non-nervous tissues its level does not change during the different developmental stages (Diaz-Nido and Avila, 1989a). Moreover, the relative ratio MAP1B/tubulin is constant in all the tissues tested, which is not the case for other MAPs (Diaz-Nido and Avila, 1989b). In conclusion, MAP1B seems to play a general role in the promotion of microtubule assembly in most cells from all mammalian tissues. Centrosomal staining by antibodies directed against MAP1B has been described in cells of organisms ranging from *Xenopus* (this work) to human (Vallee et al., 1984, 1986; Diaz-Nido and Avila, 1989a). In view of these data, an important role for MAP1B or MAP1B-related proteins in the promotion of microtubule nucleation in mammalian cells can be proposed. As hypothesized by Oakley (1992), γ-tubulin could be the initiator of tubulin assembly at the centrosome behaving as a 'nucleating cap' for the binding of tubulin dimers. The centrosomal protein related to MAP1B, could then act as a microtubule nucleation promoting factor by lowering the critical concentration for polymer assembly. Finally, soluble MAPs (like MAP4) probably act on the various parameters of microtubule dynamics.

In the present study we have also shown that a MAP1B phosphorylated epitope is located to the centrosomal region in tissue culture cells and in purified centrosomes, and that a kinase activity that could be related to casein kinase II is present in centrosomes. This work suggests that one of the in vitro substrates of this activity is the centrosomal protein related to MAP1B. These observations argue in favor of a role for phosphorylation in the regulation of the putative microtubule nucleating activity of the centrosomal MAP1B-related protein, in vivo. This is currently under investigation. As an example, it has been previously reported that brain MAP1B is phosphorylated by a casein kinase II-related enzyme and that

this phosphorylation is correlated with increased microtubule assembly during axonal outgrowth (Diaz-Nido et al., 1988). This raises the possibility that phosphorylation of the centrosomal MAP1B-related protein could be one of the mechanisms by which the intrinsic microtubule nucleating activity of centrosomes is regulated.

In conclusion, it is tempting to speculate that in vivo, the apparent nucleating activity of centrosomes in a given cell and at a given stage of the cell cycle is the result of a combination of 3 factors: (i) the presence in the pericentriolar material of MAPs like MAP1B that could promote microtubule nucleation locally; (ii) the cytoplasmic concentration in soluble MAPs capable of modulating the dynamic instability of microtubules (Bré et al., 1987); and (iii) the phosphorylation status of centrosome-associated and soluble MAPs that could regulate microtubule nucleation and dynamics (Vandre et al., 1986; Verde et al., 1990; Buendia et al., 1992).

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