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The Pool of MAP Kinase Associated with Microtubules Is Small but Constitutively Active

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Mitogen-activated protein kinase (MAPK) is activated by many kinds of stimuli and plays an important role in integrating signal transduction cascades. MAPK is present abundantly in brain, where we have studied its association with microtubules. Immunofluorescence of primary hippocampal neurons revealed that MAPK staining co-localized with microtubules and biochemical analyses showed that MAPK co-purified with microtubules. Approximately 4% of MAPK in cytosolic extracts was associated with microtubules, where it was associated with both tubulin and microtubule-associated proteins (MAPs) fractions. Further fractionation of MAPs suggested that a portion of MAPK is associated with MAP2. An association with MAP2 was also demonstrated by co-immunoprecipitation and in vitro binding experiments. A similar association was shown for the juvenile MAP2 isoform, MAP2C. The pool of MAPK associated with microtubules had a higher activity relative to the nonassociated pool in both brain and proliferating PC12 cells. Although MAPK was activated by nerve growth factor in PC12 cells, the activity of microtubule-associated MAPK did not further increase. These results raise the possibility that microtubule-associated MAPK operates through constitutive phosphorylation activity to regulate microtubule function in neurons.

INTRODUCTION

In mammalian cells, there are two major species of mitogen-activated protein kinase (MAPK)[1] known as extracellular signal-regulated kinases (ERKs), designated ERK1 (44 kDa) and ERK2 (42 kDa) (Boulton and Cobb, 1991). Other members of this family have also been described (Pelech and Sanghera, 1992). MAPK integrates multiple intracellular signals following activation by various growth factors in a signal transduction cascade that begins with ligand binding to a receptor tyrosine kinase or a G-protein coupled receptor (for reviews see Johnson and Vaillancourt, 1994; Seger and Krebs, 1995). Many types of stimuli, such as hormones, cytokines, calcium, and stress, can activate MAPK (Seger and Krebs, 1995). The MAPK pathways involve sequential protein kinase reactions, which are conserved from yeast to mammalian cells. Unique to MAPK activation is the requirement for the dual phosphorylation of both threonine and tyrosine in the TEY sequence of kinase subdomain VIII (Anderson *et al.*, 1990). Activation is catalyzed by MAPK kinase (MEK) (Ahn *et al.*, 1992), which is capable of phosphorylating both of these residues. Once activated the preferred substrate motif for MAPK is a serine or threonine residue followed by a proline (Davis, 1993). MAPK operates in a broad spectrum of cellular events including proliferation, differentiation, osmotic responses, and cell cycle regulation. Evidence for the essential role of MAPK in these processes has been obtained recently using mutated genes (Pages *et al.*, 1993; Brunner *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1994).

Given this central role of MAPK, it is surprising that its subcellular localization within the cytoplasm is not

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[1] Abbreviations used: DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; MAP, microtubule-associated protein; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NGF, nerve growth factor; PBS, phosphate-buffered saline; PP, protein phosphatase; PVDF, polyvinylidene difluoride.

well established. A general principle in cell activation is the translocation of a messenger/effector molecule to the nucleus as part of the signaling cascade (reviewed in Mochly-Rosen, 1995), and indeed, MAPK translocation to the nucleus (Chen *et al.*, 1992; Traverse *et al.*, 1992) places the kinase in proximity to many of its principal substrates, such as the transcription factors, elk-1/p62^{TCF} (Gille *et al.*, 1992; Marais *et al.*, 1993), c-jun (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991), c-myc (Alvarez *et al.*, 1991), NF-IL-6 (Nakajima *et al.*, 1993), TAL1 (Cheng *et al.*, 1993), and RNA polymerase II (Dubois *et al.*, 1994). However a portion of the kinase remains in the cytoplasm, and cytoplasmic substrates for the kinase are present in a diversity of cytoplasmic compartments. The function of substrate phosphorylation by MAPK in cytoplasm is perhaps best understood for pp90^{rsk} (Sturgill *et al.*, 1988; Chung *et al.*, 1991), which is activated by MAPK to inhibit glycogen synthase kinase 3 (GSK3) function or activate protein phosphatase 1 (PP1) activity, and consequently regulate glycogen synthesis (Dent *et al.*, 1990; Lavoinne *et al.*, 1991; Sutherland *et al.*, 1993; Eldar-Finkelman *et al.*, 1995). Cytosolic phospholipase A_2 is also activated by MAPK phosphorylation and appears to mediate agonist-stimulated release of arachidonic acid (Lin *et al.*, 1993). Less understood are the consequences of MAPK phosphorylation of EGF receptor (Northwood *et al.*, 1991; Takishima *et al.*, 1991), Raf-1 (Anderson *et al.*, 1991; Lee *et al.*, 1992), stathmin (Leighton *et al.*, 1993), and MAPK kinase (Matsuda *et al.*, 1993).

The neuronal microtubule-associated proteins (MAPs), MAP2 and tau, are also putative substrates for MAPK. Both incorporate significant amounts of phosphate following in vitro phosphorylation with MAPK (Ray and Sturgill, 1987; Drewes *et al.*, 1992), and some of the sites of incorporation correspond to in vivo phosphorylation sites (Watanabe *et al.*, 1993; Sanchez *et al.*, 1995). However, the association of MAPK with microtubules is not well characterized. Mandelkow *et al.* (1992) and Lu *et al.* (1993) showed that MAPK was co-precipitated through cycles of microtubule polymerization, but there is a contradiction about the phosphotyrosine staining of MAPK between the two reports; neither has measured the real activity of MAPK. Reszka *et al.* (1995) found a pool of MAPK associated with microtubules in NIH 3T3 cells and provided an explanation why many other analyses found only a diffuse presence of MAPK throughout the cytoplasm (Chen *et al.*, 1992; Traverse *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). Their work, however, provided evidence only for a cytoskeletal association of MAPK.

The binding of MAPK to microtubules would serve as an attractive means for local regulation of MAP binding to microtubules and microtubule assembly. In vitro experiments showed that MAP phosphorylation by MAPK increases the dynamic instability of microtubules (Drechsel *et al.*, 1992; Hoshi *et al.*, 1992). The degree and sites of MAP phosphorylation affect their ability to polymerize microtubules by altering the binding characteristics of the MAPs to the microtubules (Sloboda *et al.*, 1975; Lindwall and Cole, 1984; Gustke *et al.*, 1992). MAP phosphorylation affects microtubule dynamics by modulating these binding properties (Drechsel *et al.*, 1992). The very large number of MAP phosphorylation states suggest a very finely tuned regulation of these dynamic properties.

Some protein kinases and phosphatases putatively involved in the phosphorylation of MAPs are reported to bind to microtubules (Vallee *et al.*, 1981; Rattner *et al.*, 1990; Sontag *et al.*, 1995). In this study, we have investigated the association of MAPK with microtubules in neurons. Our results showed that a pool of MAPK is localized to microtubules via an association with MAP2 and tubulin, and this pool has a higher baseline kinase activity than the nonassociated fraction.

MATERIALS AND METHODS

Cell Cultures

Neuronal cultures were prepared from the hippocampi of embryonic day 18 rats as described (Goslin and Banker, 1991). Briefly, cells from the dissected hippocampi were dissociated by trypsinization (0.25% for 15 min at 37°C) followed by trituration with fire-polished Pasteur pipettes. The cells were plated at a density of 100,000 cells/60-mm culture dish on poly-L-lysine coated (1 mg/ml) glass coverslips in minimum essential medium (MEM) with 10% horse serum. After 2 h, the medium was changed to MEM with N2 supplements (Bottenstein and Sato, 1979), ovalbumin (0.1%), and sodium pyruvate (1 mM) that had been conditioned in cultures of astroglial cells for 24 h, and coverslips plated with neurons were cocultured with astroglia.

NIH 3T3 cells were maintained in DMEM containing 10% fetal calf serum. PC12 pheochromocytoma cells were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum. Treatments with nerve growth factor (NGF) were performed using 50 ng/ml NGF (Promega Biotech, Madison, WI).

Immunocytochemistry

For immunostaining, the cultures were fixed for 20 min with warm 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.12 M sucrose, permeabilized in 0.3% Triton X-100 for 5 min at room temperature, and rinsed in PBS. In some experiments, the cells were extracted for 1–2 min in PHEM (60 mM piperazine-*N*,*N*′-bis(ethansulfonic acid) [Pipes], 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 0.2% Triton X-100, 0.1% dimethyl sulfoxide (DMSO), and 10 μM taxol (Brown *et al.*, 1992), rinsed in the same buffer without Triton, and then fixed. The cultures were then incubated with 10% goat serum in PBS for 1 h and exposed to primary antibodies overnight at 4°C. After several washes in PBS, the cells were incubated for 1 h with fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Labs., West Grove, PA). Control experiments were performed in which primary antibodies were omitted. For some double labeling experiments, samples were scanned by a laser confocal microscope (Bio-Rad Laboratories, Richmond, CA).

To depolymerize microtubules, cells were treated with colchicine (20 μg/ml) (Sigma Chemical, St. Louis, MO) or nocodazole (10

μg/ml) (Sigma) in culture media for 1 h before fixation. Stock solutions of colchicine and nocodazole were made up in DMSO and ethanol, respectively, and the concentration of these solvents was diluted below 0.1% in the culture media, as not to be toxic to neurons. DMSO or ethanol (0.1%) was added to the culture medium of control cells.

Purification of Proteins

Protease inhibitors used in this study were 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 1 μg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml *N*α-p-tosyl-L-lysinechloromethyl ketone, and 0.1 mM diisopropyl fluorophosphate. Protein phosphatase inhibitors used were 20 mM sodium pyrophosphate, 20 mM NaF, 1 mM sodium orthovanadate, and 0.5 mM okadaic acid.

Microtubules were prepared from 6-wk old rat brain through three cycles of temperature-dependent assembly-disassembly purification as described (Ihara *et al.*, 1979). Microtubules were also prepared essentially according to the taxol method of Vallee (1982). To dissociate MAPs from microtubules, microtubule pellets were resuspended in buffer A (0.1 M MES, pH 6.5, 1 mM EGTA, 1 mM $MgCl_2$) containing 1 mM GTP and 20 μM taxol, and NaCl was added to 0.35 M. After incubation at 37°C for 10 min, the solution was centrifuged at 30,000 × *g* for 25 min, leaving the MAPs in the supernatant (Vallee, 1982).

The MAP fraction was submitted to gel filtration on a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 20 mM Pipes, pH 6.9, 0.1 M NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM GTP at a flow rate of 0.3 ml/min and fractions of 0.3 ml were collected. The MAP fraction was also loaded on a phosphocellulose (P11) (Whatman, Maidstone, UK) column equilibrated with buffer A and eluted with buffer A containing 0.1–0.5 M NaCl in step-wise increments. As a control experiment, microtubule-depleted supernatants were fractionated with the same procedures.

MAP2C and tau were purified from Sf9 cells infected with the recombinant baculovirus expressing MAP2C and tau, respectively (Knops *et al.*, 1991; LeClerc *et al.*, 1993). Three days after infection, cells were harvested and homogenized in buffer A. The homogenates were spun at 500,000 × *g* for 15 min. After addition of 0.75 M NaCl and 2% β-mercaptoethanol, the supernatants were heated to 97°C for 10 min and cleared by centrifugation at 500,000 × *g* for 20 min.

The plasmid pCMV5/ERK2 was a generous gift from Melanie Cobb (University of Texas Southwestern Medical Center, Dallas, TX) and expresses ERK2 tagged with hexahistidine in eukaryotic cells (Sontag *et al.*, 1993). NIH 3T3 cells plated 1.6×10^6 per 100-mm diameter dish were transfected with 16 μg of pCMV5/ERK2 using lipofectamine (Life Technologies, Grand Island, NY) as described by the manufacturer. After approximately 50 h, expressed ERK2 was purified with Ni^{2+}-NTA-agarose (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. Highly purified ERK2 was generated from *Escherichia coli* transformed with histidine-tagged ERK2 expression plasmid (Robbins *et al.*, 1993) and provided by M. Cobb.

Immunoprecipitation

For co-immunoprecipitation, newborn (1-day old) or adult (10-wk old) rat brains were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5% Triton X-100, and various protease and protein phosphatase inhibitors as described above) and the homogenates were centrifuged at 500,000 × *g* for 15 min on a TL 100.3 microcentrifuge (Beckman, Palo Alto, CA). The supernatants were precleared with protein G- or protein A-Sepharose and then incubated for 3 h at 4°C with anti-MAP2 (HM2, 1:20) or anti-MAPK (R1, 1:100; C14, 1:20) antibodies. In some experiments, rabbit IgG or mouse IgG was used as control. Immune complexes were brought down by protein G-Sepharose (Pharmacia) or protein A-Sepharose (Sigma) and washed four times in lysis buffer. Precipitated proteins were boiled in SDS-PAGE sample buffer and separated by SDS-PAGE followed by immunoblotting.

In Vitro Binding Assay

For in vitro binding of protein to an immobilized second protein, MAP2C and tau purified from Sf9 cells or ERK2 purified from transfected NIH 3T3 cells were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). Filters were blocked with 5% dried skimmed milk and then incubated either with 0.15 μM ERK2 purified from *E. coli*, MAP2C, or tau in lysis buffer for 5 h at 4°C. After extensive washing, binding proteins were detected by immunoblotting using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL). A 30-fold molar excess of competitor protein was used in binding reaction when appropriate.

Assay of MAPK Activity

MAPK activity was measured in immune complexes basically as described (Greenberg *et al.*, 1994). PC12 cells were exposed to NGF (50 ng/ml) for 10 min and rinsed twice with cold PBS containing 1 mM sodium orthovanadate. Microtubules were prepared from NGF-treated or untreated PC12 cells or rat brain using taxol as described above and protein fractions were normalized for protein concentration. Ten microliters of anti-MAPK (C14) was added to IP buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.1% Briji-35, 0.1% deoxycholate, 1 mM benzamidine, various protease and protein phosphatase inhibitors) containing equal amounts of protein and precipitated with protein A-Sepharose. Immunoprecipitates were added to 30 μl of kinase buffer (30 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM benzamidine, 15 μg myelin basic protein [MBP], 50 μM [γ-^{32}P]ATP [1 μCi]) and incubated at 30°C for 30 min. Reactions were terminated by boiling in SDS-PAGE sample buffer. The samples were resolved on 15% polyacrylamide gels and quantified by PhosphoImager analysis (Molecular Dynamics, Sunnyvale, CA).

Antibodies and Immunoblotting

Immunoblotting was performed on a PVDF membrane. The bound antibodies were detected by alkaline phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch) or enhanced chemiluminescence. For quantitative study, ^{125}I-labeled anti-rabbit IgG (Amersham) was used as a secondary antibody and the bands were quantified with PhosphoImager. Dot immunobinding was also performed.

Antibodies used against MAPK were R2 (Upstate Biotechnology, Lake Placid, NY; 1:700), R1 (Upstate Biotechnology; 1:500), C14 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:300), and anti-MAPK (Zymed, South San Francisco, CA; 1:500). R2 and R1 recognize both ERK1 and ERK2 in SDS-denatured lysates, but only ERK1 under native conditions. C14 recognizes ERK2 and, to a lesser extent, ERK1 in both denatured and nondenatured conditions. Antibodies used against MAP2, tau, MAP1A, MAP5, and tubulin were HM2 (Sigma; 1:500), 5E2 (1:1000) (Kosik *et al.*, 1988), HM1 (Sigma; 1:500), AA6 (Sigma; 1:100), and DM1A (Sigma; 1:500), respectively. HM2 recognized both MAP2 and MAP2C. These antibodies did not cross-react with each other in the immunoblotting analysis.

For immunocytochemistry, R2, C14, and DM1A were used at a dilution of 1:20, 1:50, and 1:200, respectively. Other antibodies used were AP14 (1:100), which specifically recognizes MAP2 and was provided by Lester Binder, and anti-neurofilament 160 kDa (Sigma, 1:40; Amersham, 1:5). Rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) was also used.

RESULTS

Immunolocalization of MAPK in Cultured Hippocampal Neurons

MAPK immunoreactivity was observed throughout the cytoplasm at all stages of neuronal development in culture. In young neurons, MAPK antibody stained both the cytoplasm and nucleus diffusely. Although all the minor neurites stained, the proximal portion of the neurites reacted most intensely. In mature neurons cultured for 19 days, which have established a clear morphological distinction between dendrites and axon, MAPK immunoreactivity was present in all the processes, both dendrites and axons.

Double labeling with the MAPK antibody and a tubulin antibody suggested some degree of co-localization. The finding of co-localization was most convincing within certain regions of the growth cone (Figure 1, A and B) due to its flattened structure, in which single microtubule bundles can be discriminated. Colocalization was best appreciated for those microtubules that assumed a looping configuration because MAPK staining was considerably less intense immediately adjacent to the microtubules. Both of these patterns differed from rhodamine-phalloidin labeling of actin filaments. The most distal region of neurites, an actin-rich site that is intensely stained with rhodamine-phalloidin and excludes tubulin-immunoreactivity, has detectable, albeit less intense, MAPK staining (Figure 1B).

Neurons after 19 days in culture were extracted with 0.2% Triton X-100 before fixation. This procedure leaves most cytoskeletal elements intact, including microtubules when appropriate buffers are used. Following extraction, intact microtubules were still present as shown by tubulin staining. MAPK immunoreactivity was also retained, indicating that MAPK was tightly associated with the Triton-resistant fraction (see also Reszka *et al.*, 1995). Persistent staining of all the processes with MAPK antibodies demonstrated the presence of a Triton-resistant MAPK pool in both axon and dendrites. Double staining with tubulin and MAPK antibodies and analysis of the images by confocal microscopy showed that MAPK precisely coincided with the microtubules, although the relative intensities of the two signals differed in some regions of the cytoplasm; anti-tubulin strongly stained a region close to the nucleus, which probably represents the microtubule organizing center. MAPK staining was more uniform over most of the cytoplasm (our unpublished observations). When neurons cultured for 2 days were extracted with Triton, MAPK staining was also preserved. Because neurofilament proteins are not expressed until 4 days in culture (Shaw *et al.*, 1985), it is unlikely that MAPK is anchored by neurofilaments in those neurons. Taken together, these results indicate that one pool of MAPK is associated with microtubules in neurons. These results confirmed the study of Fiore *et al.* (1993), which had shown the staining of microtubules with MAPK antibody in brain sections by immuno-electron microscopy. In addition, our results also showed the presence of MAPK in both axons and dendrites. Probably because of the strong fixation for histochemistry or because they used the antibody specific for p42 MAPK, they failed to detect the MAPK staining in axons.

To examine the influence of microtubule-disrupting reagents on the MAPK distribution, neurons were treated with colchicine or nocodazole for 1 h before

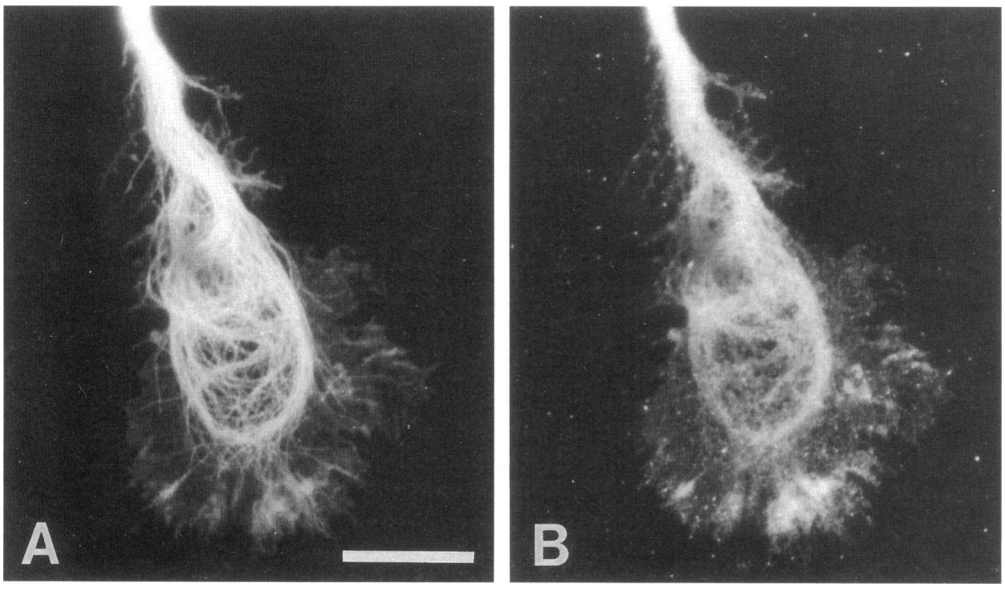

Figure 1. Double labeling of hippocampal neurons with monoclonal tubulin (A) and polyclonal MAPK (R2) (B) antibodies 1 day after plating. Note that in the growth cones of young neurons, MAPK immunostaining co-localized with tubulin staining. Bar, 10 μm.

extraction and fixation. Although the caliber of the processes was significantly reduced and the intensity of tubulin staining became weak, drug-stable microtubules remained intact after these treatments. MAPK staining was also retained and continued to colocalize with the drug-stable microtubules, suggesting that a portion of MAPK may associate with drug-stable microtubules.

MAPK Is Associated with Microtubules

To verify the association of MAPK with microtubules biochemically, microtubules and microtubule-binding protein were purified from high speed extracts of rat brain using two standard methods and analyzed by immunoblotting. Co-purification through repeated cycles of temperature-dependent microtubule assembly and disassembly showed that a significant portion of MAPK associated with microtubules even in pellets from the third cycle of assembly, the usual criterion for defining a MAP. Both ERK1 and ERK2 isoforms co-purified with microtubules. This is consistent with previous findings (Mandelkow *et al.*, 1992; Lu *et al.*, 1993). Co-sedimentation of assembled microtubules using taxol also brought down a readily detectable fraction of the total MAPK (Figure 2, lanes 1–3). Quantitative analysis using ^{125}I-labeled secondary antibody revealed that 4% of the MAPK in the cytosolic extract was co-purified with taxol-prepared microtubules in vitro. When we extracted the neuronal culture with Triton/PHEM buffer and analyzed quantitatively, 25% of MAPK was present in the cytoskeletal fractions. This proportion is similar to the result by Reszka *et al.* (1995) using NIH 3T3 cells and higher than that obtained by the purification of microtubules. However, this "cytoskeleton" fraction includes not only microtubules, but the Triton-resistant membrane fraction that includes caveolae, an organelle that also contains MAPK (Lisanti *et al.*, 1994). And some MAPK may be associated with other cytoskeletal elements. When the MAP fraction was separated from the tubulin fraction by increasing the salt concentration in the presence of taxol and GTP, MAPK distributed to both fractions (Figure 2, lanes 4 and 5). However, the isoform preferences of ERK1 and ERK2 differed; ERK1 was relatively enriched in the tubulin fraction, and ERK2 in the MAP fraction.

The MAP fraction was further fractionated using a gel-filtration column under native conditions to examine which MAP or MAPs the MAPK accompanies (Figure 3A). Although a very small amount of MAPK was present in almost all the fractions, a larger amount was eluted from the column at the elution time expected from the molecular weight of MAPK. ERK1 eluted a little earlier than ERK2. In addition, some MAPK was eluted at a higher molecular size, in the same fraction as MAP2. Those fractions contained

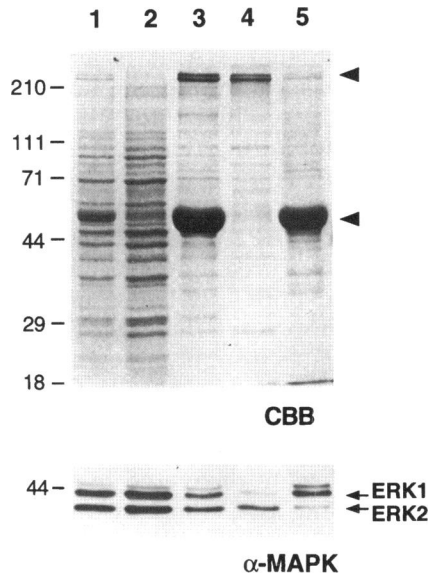

Figure 2. Co-polymerization of MAPK with microtubules. Microtubules were prepared from rat brain by the taxol method (lanes 1–3). Purified microtubules were separated into MAPs and tubulin fractions in the presence of 0.35 M NaCl (lanes 4 and 5) (see MATERIALS AND METHODS). The same amounts of protein (15 μg) (lanes 1–3) or equal volume of aliquots (lanes 3–5) were subjected to SDS-PAGE and stained with Coomassie brilliant blue (upper panel). MAPK was detected by immunoblotting with mixed MAPK antibodies (R2 plus C14) (lower panel). The 45-kDa band migrating just above ERK1 seems to correspond to ERK4 (see Boulton and Cobb, 1991). Lane 1, cytosolic extract; lane 2, microtubule-depleted supernatant; lane 3, microtubule pellets; lane 4, MAPs fraction eluted from microtubules by salt extraction; and lane 5, microtubule pellets obtained after salt extraction. Positions of ERK1 and ERK2 (arrows), high molecular weight MAPs (upper arrowheads) and tubulin (lower arrowheads) are indicated. Positions of molecular weight standards are shown at the left of each panel (in kDa).

high molecular weight MAPs including MAP1A as well as MAP2. To resolve the MAPK from co-eluting MAPs, we separated the MAPs fraction also with a cation-exchange column, a phosphocellulose column. When a microtubule-depleted supernatant was loaded on the column, most of the MAPK was eluted with less than 0.1 M of NaCl, and a small amount of MAPK was eluted with 0.2 M NaCl; ERK1 was present mainly in the flowthrough fraction, and ERK2 was eluted at a higher salt concentration after ERK1 (Figure 3B). This finding is consistent with previous reports (Boulton and Cobb, 1991; Boulton *et al.*, 1991). An immunoblot analysis of the MAP fraction applied onto and eluted from the column showed that the elution position of a part of MAPK shifted to 0.3 M NaCl eluates, where MAP2, but neither tau nor MAP1A, was eluted from the column (Figure 3C). That a portion of MAPK co-purified with MAP2 suggested that some MAPK binds to MAP2.

Immunoprecipitation of MAPK with MAP2

To investigate whether MAPK is associated with MAP2, MAP2 was immunoprecipitated from rat brain extracts and analyzed for the presence of MAPK by immunoblotting (Figure 4A). The mature MAP2 isoform was immunoprecipitated from adult rat brain; the juvenile isoform, MAP2C, and a small amount of mature MAP2 were immunoprecipitated from newborn rats. Three independent MAPK antibodies recognized both ERK1 and ERK2 bands in the MAP2 immunoprecipitates (Figure 4A and legend). The precipitates from both newborn and adult rat extracts stained with anti-MAPK. Co-immnoprecipitation experiments in the reverse way using newborn rat brains showed that MAP2C and a small amount of MAP2 were recovered in MAPK immunoprecipitates using two different antibodies (Figure 4B); an ERK1 antibody (R1) and the C14 antibody, which prefers ERK2, both precipitated MAP2. Because MAP2C, as well as mature MAP2, were co-immunoprecipitated with MAPK, a direct or indirect interaction between these molecules must occur within their common region. The ratio of MAP2C and MAP2 in the immunoprecipitates reflected that in the extracts. Tau, MAP1A, or MAP5 were not detected in the MAPK immunoprecipitates by immunoblotting. These results indicate that MAPK is associated with MAP2 and MAP2C, but not with tau or MAP1.

MAPK Interacts with MAP2C In Vitro

To determine whether the association of MAPK with MAP2 is a direct interaction, we performed in vitro binding studies. MAP2C was used in this experiment, because MAP2C is a shortened form of MAP2 in which amino acids 148 to 1518 are not present in this alternatively spliced juvenile isoform (Papandrikopoulou *et al.*, 1989). MAPK was purified from NIH 3T3 transfectants with Ni^{2+}-NTA agarose, separated by SDS-PAGE, and electroblotted onto PVDF membranes. To detect direct binding of MAP2C to MAPK, the blot was incubated with MAP2C purified from Sf9 cells, and bound MAP2C was detected with anti-MAP2 antibodies. As shown in Figure 5A, MAP2C bound to MAPK immobilized onto the membrane. On the other hand, tau did not bind.

When MAP2C and tau were immobilized to the membrane and overlaid with MAPK followed by immunodetection with MAPK antibody, MAPK bound to MAP2C, but not to tau (Figure 5B). In the absence of the MAPK protein, anti-MAPK antibody did not react with the MAP2C band. To assess the specificity of the binding between MAPK and MAP2C, competition experiments were performed. In the presence of excess molar of MAP2C in the binding reaction, the binding of MAPK to immobilized MAP2C was prevented (Figure 5B, left panel). From these results, we conclude that MAPK binds to MAP2C directly in vitro. Furthermore, MAPK binds to MAP2 at either the amino or

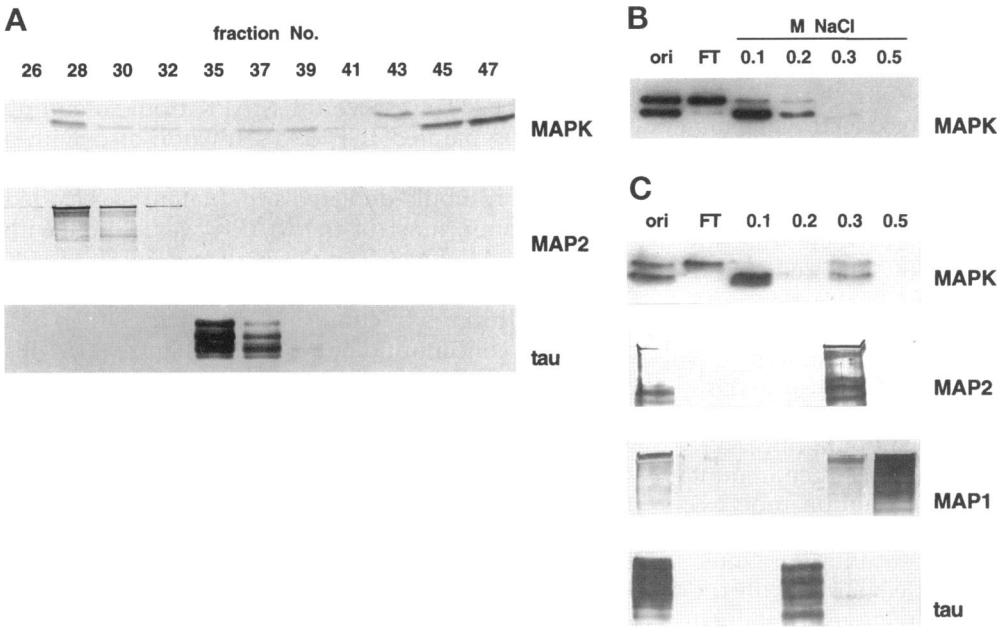

Figure 3. Immunoblotting after further fractionation of the MAPs and co-distribution of MAPK. The MAPs fraction prepared from rat brain was fractionated on a size-exclusion column (superose 12 HR10/30) (A). The fraction was also applied onto a phosphocellulose column from which the bound proteins were eluted in a step-wise gradient with 0.1–0.5 M NaCl as indicated at the top of each lane (C). As a control, microtubule-depleted supernatant was loaded on a phosphocellulose column and eluted in the same procedure (B). Ori, protein fraction applied onto a column; and FT, flow through fraction that did not bind to a column. Aliquots from each fraction were immunoblotted with MAPK, MAP2, MAP1A (MAP1), and tau antibodies. Co-fractionation of a portion of MAPK with MAP2 suggests the physical association between MAPK and MAP2.

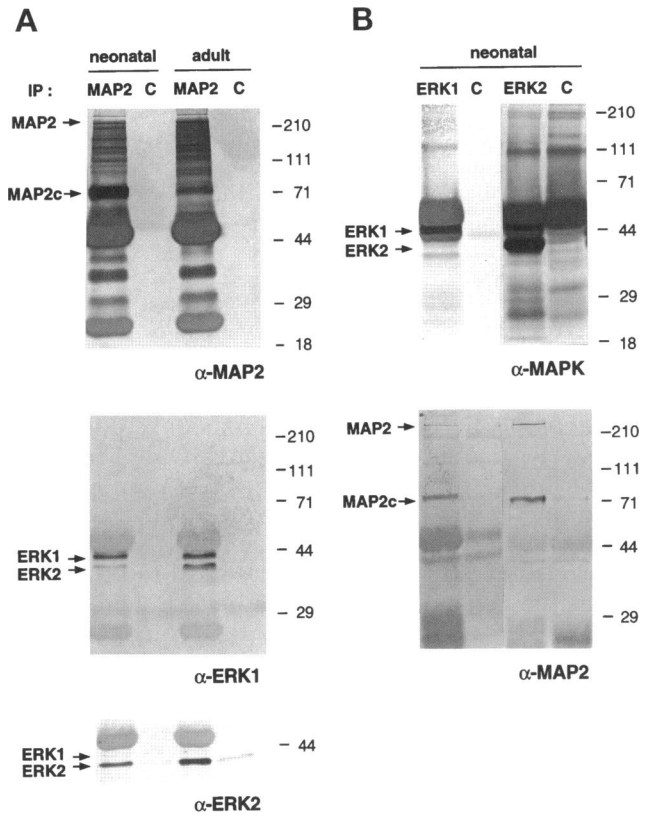

Figure 4. Co-immunoprecipitation of MAPK with MAP2 and MAP2C. Extracts from neonatal and adult rat brain were subjected to immunoprecipitation with MAP2 (A) or MAPK antibodies (B), R1 (ERK1), or C14 (ERK2). Bound proteins were analyzed by immunoblotting with the antibodies indicated at the bottom of the each panel. Control experiments were performed in the absence of antibodies or in the presence of mouse IgG or rabbit IgG (lanes indicated by C). Ig heavy chain (~55 kDa) and light chain (~23 kDa) contained in the immune complex were also stained with secondary antibodies in the upper panels. Note that MAP2 immunoprecipitates were stained by MAPK antibodies and that MAPK immunoprecipitates were stained by MAP2 antibody. Anti-MAPK staining of MAP2 immunoprecipitates also showed the same results.

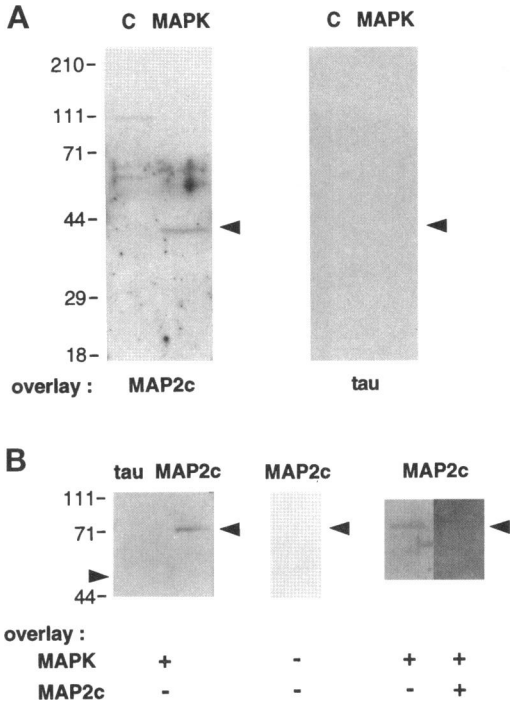

Figure 5. In vitro binding between MAPK and MAP2C. (A) NIH 3T3 cells were transfected with either MAPK expression clone (MAPK) or vector alone (C). The tagged protein was purified from transfectants with Ni^{2+}-NTA-agarose, separated on an SDS gel, and electroblotted onto a PVDF membrane. The filters were incubated with MAP2C (left panel) or tau (right panel) purified from baculovirus-infected Sf9 cells. The binding was detected with either MAP2 or tau antibodies. The position of MAPK is indicated by arrowheads. (B) Tau and MAP2C were immobilized on a PVDF membrane. The blots were incubated with (left panel) or without (central panel) MAPK, which was purified from *E. coli* and the bound MAPK was detected with MAPK antibodies. For the competition of the MAPK binding with immobilized MAP2C, excess molar of MAP2C was added to the reaction buffer (right panel). The positions of tau and MAP2C were indicated by arrowheads in each lane. Note that MAPK interacts with MAP2C, but not with tau in vitro.

carboxy end of the protein because the middle region is not present in MAP2C, and yet binding still occurs.

The Activity of MAPK on Microtubules

We next asked whether the MAPK pool associated with microtubules is active, and if so, under what circumstances. To assess this, we first used PC12 cells, because the activation of MAPK following stimulation with NGF is well characterized and includes the reorganization of microtubules along with neurite outgrowth.

Microtubules were purified from NGF-stimulated and unstimulated PC12 cells. MAPK was co-purified with microtubules in PC12 cells as well as in brain as shown by the immunoblot analysis (our unpublished observations). Although the yield of microtubules from PC12 cells was lower than that from brain, MAPK was also relatively enriched in the microtubule pellets from PC12 cells. Thus, a similar proportion of MAPK was associated with microtubules in PC12 cells.

The activity of MAPK in the total cell lysates, the microtubule fraction, and the microtubule-depleted supernatants was measured by an immunocomplex assay using MBP as a substrate. In unstimulated PC12 cells, there was a low basal level of MAPK activity in cell lysates (Figure 6A). Interestingly, MAPK activity in the microtubule fraction was much higher than that found in the residual microtubule-depleted supernatant. Following the exposure of PC12 cells to NGF, MAPK in cell lysates was activated several fold. MAPK activity in the microtubule-depleted superna-

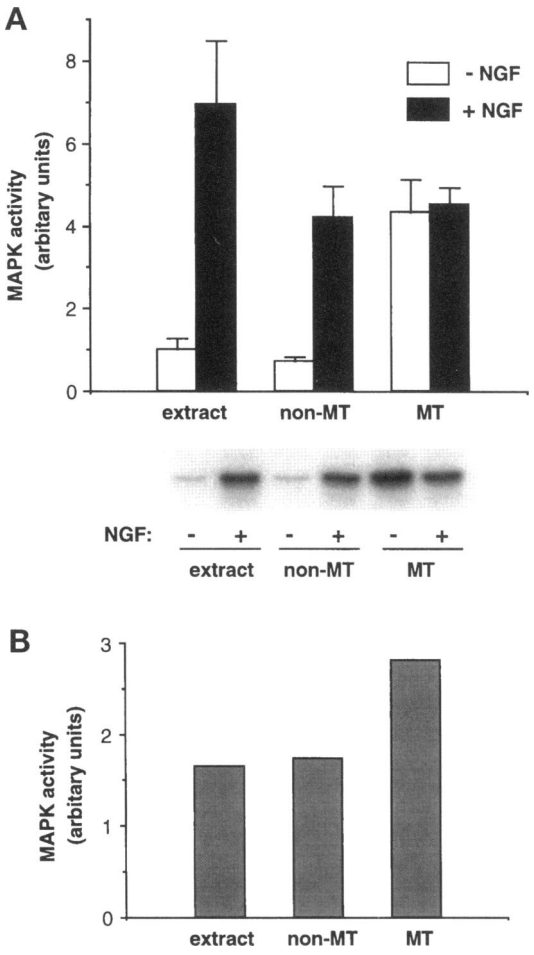

Figure 6. The activity of MAPK associated with microtubules in PC12 cells (A) and in brain (B). (A) PC12 cells were incubated for 10 min in the absence (open bars) or in the presence (closed bars) of NGF. MAPK was immunoprecipitated from the total cytosolic extract (extract), microtubule-depleted supernatant (non-MT), and microtubule fraction (MT) and subjected to an in vitro kinase assay with MBP as a substrate as described in MATERIALS AND METHODS. Quantification was performed by a PhosphoImager analysis. Bars represent the means ± SEM from seven independent experiments. Lower panel shows the autoradiogram of one example of such experiments. (B) MAPK activity in immune complexes was measured in the total cytosolic extract, microtubule-depleted supernatant, and microtubule fraction prepared from rat brain. Data shown are the means of two separate experiments.

tant was also stimulated by NGF treatments, although the extent of activation was less than that in cell lysates because of the intervening purification steps. In contrast, the level of MAPK activity in the microtubule fraction did not change upon stimulation with NGF; it retained a constitutive level of activity. The control experiment without addition of MAPK antibodies showed negligible levels of kinase activity in all the fractions. These results identify a pool of constitutively active MAPK whose activity does not depend on NGF stimulation.

To determine microtubule-associated MAPK activity in brain, we next measured the activity of MAPK in the microtubule fraction purified from rat brain (Figure 6B). MAPK in brain showed a moderate activity. As in the case of PC12 cells, the activity of MAPK in the microtubule fraction was more significant than those in the brain extracts or in the microtubule-depleted supernatants.

DISCUSSION

A Microtubule-associated Pool of MAPK

We have defined a small, but significant pool of MAPK associated with cytoplasmic microtubules in neural tissue. In the microtubule fraction, a portion of the MAPK is physically associated with MAP2 as demonstrated by co-purification, co-immunoprecipitation, and in vitro binding experiments. Because both mature MAP2 and MAP2C interact with MAPK, sequences shared by these molecules must represent the interactive region. cAMP-dependent protein kinase (PKA) is also associated with MAP2 through an interaction of the common amino-terminal domain of MAP2 with the regulatory subunit of PKA (Obar *et al.*, 1989; Rubino *et al.*, 1989). Therefore, one role of the long projection domain of MAPs serves to anchor cytoplasmic kinases, and thereby limit their accessibility to substrates.

The pool of MAPK associated with microtubules has a higher basal activity in proliferating PC12 cells than the MAPK in the rest of the cytosol. As expected, NGF stimulated total MAPK activity in PC12 cells, but the activity of microtubule-associated MAPK was not additionally stimulated and remained unchanged. Microtubule-associated MAPK also has a higher basal activity in brain. In both brain and PC12 cells, we normalized samples for the amount of protein. The microtubule fraction contained a lower amount of MAPK protein per unit of total protein compared with the microtubule-depleted supernatant (see Figure 2, lanes 2 and 3). Thus, the difference in the activity per mole of MAPK between these two fractions is even larger than suggested simply by the comparison between the microtubule and the microtubule-depleted supernatant shown in Figure 6. The activity of microtubule-associated MAPK did not parallel the total MAPK activity. This indicates that the MAPK activity associated with microtubules is not a nonspecific association. Since microtubule-associated MAPK is not activated even when MAPK in total cell lysate was activated, it is unlikely that activated MAPK can bind preferentially to microtubules in vitro.

Putative Substrates for Microtubule-associated MAPK

Because tubulin itself is poorly phosphorylated by MAPK in vitro (Gotoh *et al.*, 1991), MAPs are strong

candidate substrates for the microtubule-associated MAPK. Among the MAPs, MAP2 is an excellent substrate for MAPK in vitro as well as MAP1A (Tsao *et al.*, 1990). Tau is also phosphorylated by MAPK in vitro, which is notable because Alzheimer's paired helical filament tau is phosphorylated at sites that are phosphorylated by MAPK in vitro (Drewes *et al.*, 1992; Morishima-Kawashima *et al.*, 1995). However, the efficiency of tau phosphorylation by MAPK is 20-fold less when compared with MAP2 (Gotoh *et al.*, 1991). Furthermore, in the case of tau, lengthy incubations are necessary for high phosphate incorporation (Drewes *et al.*, 1992). Both MAP2 and tau are phosphorylated at multiple sites in vivo, some of which correspond to the in vitro phosphorylation sites of MAPK (Drewes *et al.*, 1992; Sanchez *et al.*, 1995).

We have shown here the association of MAPK with MAP2. Our preliminary experiments showed that the protein with the same mobility as MAP2 by SDS-PAGE was phosphorylated in the MAPK immunocomplex assay from a brain microtubule fraction without the addition of the exogenous substrate (MBP). This observation supports the finding of an association between MAPK and MAP2, as well as previous studies that showed that MAP2 can be phosphorylated by MAPK in vitro. Together these data strongly suggest that MAP2 is an vivo substrate for MAPK. Our results do not exclude the possibility that tau may also be a substrate for MAPK in vivo, but tau does not appear to anchor this kinase. Proximity of an enzyme to its substrate would allow immediate phosphorylation without diffusion; anchoring limits encounters with inappropriate substrates.

Functional distinctions or substrate specificities among the members of the MAPK family are unknown. ERK1 shows a preferential association with the NGF receptor $gp140^{prototrk}$ (Loeb *et al.*, 1992), there is relatively more ERK 1 in the nucleus of chicken fibroblasts and hepatoma cells (Sanghera *et al.*, 1992), and one human isoform of ERK2 designated $p41^{mapk}$ is localized at the ruffling membrane of the cell surface after serum stimulation (Gonzalez *et al.*, 1993). Within the microtubule fractions the relative proportions of ERK1 and ERK2 differed (Figure 2). ERK1 was more abundant in the tubulin fraction, and ERK2 was more abundant in the MAP fraction. This agrees with the previous observation that showed prominent localization of ERK2 in neuronal cell bodies and dendrites (Fiore *et al.*, 1993). These differences may reflect functional distinctions between the ERKs. On the other hand, the association between ERK2 and tubulin may be relatively weak and less resistant to the high salt concentration used during separation of MAPs from the tubulin fraction. The association of ERK1 with tubulin might be stronger due to the charge differences between ERK1 and ERK2, as observed by ion-exchange chromatography.

Functions for the Microtubule-associated Pool of MAPK

In the *Xenopus* oocyte, the activation of MAPK is closely tied to the microtubule reorganization that occurs during the cell cycle. Activated MAPK induces the interphase-metaphase transition of microtubule arrays (Gotoh *et al.*, 1991). In PC12 cells, the pool of MAPK associated with the microtubules is unresponsive to NGF, and is therefore, unlikely to have a direct role in the reorganization of the cytoplasmic microtubules following NGF treatment. What then might be the function of this constitutively active pool? Growth factor stimulation of cells induces a translocation of MAPK to the nucleus (Chen *et al.*, 1992; Traverse *et al.*, 1992), but nuclear translocation does not depend on MAPK activation (Lenormand *et al.*, 1993). Therefore, microtubules may hold in reserve an active pool capable of substrate phosphorylation immediately after arrival in the nucleus. MAPK nuclear translocation does not depend upon the integrity of microtubules because NGF-induced translocation occurred after treatment of PC12 cells with colchicine (our unpublished observations).

The microtubule pool of MAPK may function to maintain MAPs, particularly MAP2, at a constant level of phosphorylation. MAPs carry many phosphates, e.g. up to 46 on MAP2 (Tsuyama *et al.*, 1987), and these phosphates turn over rapidly. Rapid turnover of the phosphates on microtubules in vivo was suggested by the report of high activity of protein phosphatase in the postmortem period (Matsuo *et al.*, 1994), and the presence of phospho-MAPs under in vivo circumstances (Tsuyama *et al.*, 1987; Watanabe *et al.*, 1993).

An exquisitely tight control over MAPs phosphorylation is crucial for setting the dynamic instability of microtubules. For example, the phosphorylation of tau by MAPK affects microtubule polymerization rates, catastrophe rates, and depolymerization rates by perturbing the affinity of tau for the microtubule lattice (Drechsel *et al.*, 1992). The phosphorylation of MAP2 and MAP4 by MAPK induces interphase-M phase transition of microtubule dynamics in the reconstitution system nucleated by centrosomes (Hoshi *et al.*, 1992). Thus, the rapid phosphate exchange may regulate microtubule dynamics in vivo. Microtubule-associated MAPK could serve to maintain a phosphate equilibrium that at any given moment is undergoing rapid exchange.

Another possibility is that a microtubule-associated MAPK may have a role in microtubule-based organelle transport or membrane trafficking (Kelly, 1990). The phosphatase inhibitor okadaic acid inhibits the fusion of endocytotic vesicles (Woodman *et al.*, 1992) and induces membrane transport (Hamm-Alvarez *et al.*, 1993). The phosphorylation of kinesin or kinesin-associated proteins may modulate the binding

of kinesin to membrane organelles or its motor activity (Sato-Yoshitake *et al.*, 1992; McIlvain *et al.*, 1994).

Several protein kinases and phosphatases putatively involved in the phosphorylation of MAPs appear to bind to microtubules. These enzymes include PKA (Vallee *et al.*, 1981; Theurkauf and Vallee, 1982), cdc2 kinase (Rattner *et al.*, 1990; Tombes *et al.*, 1991), GSK3 (Ishiguro *et al.*, 1992, 1993), cyclin-dependent protein kinase 5 (cdk5) (Ishiguro *et al.*, 1992; Kobayashi *et al.*, 1993), protein phosphatase 2A (PP2A) (Sontag *et al.*, 1995), and MAPK (Mandelkow *et al.*, 1992; Reszka *et al.*, 1995). At the beginning of this study, we screened protein kinases and protein phosphatases that are associated with microtubules through an association with MAP2 or tau. From the results of co-immunoprecipitation experiments, MAPK, MEK1, protein kinase C, casein kinase II, protein phosphatase 2B (PP2B), PP2A, Raf1, and $pp60^{src}$ could be precipitated with MAP2 antibody and, MEK1, MAPK, PP2A, fyn, and lck, with tau antibody. Immunoblot analysis of purified microtubules showed that MAPK, MEK1, MEK2, cdk5, cdc2, PP2A, PP2B, PP1, and Raf1 were detected in microtubule pellets. Although the significance of these associations has not yet been elucidated, these enzymes represent candidate proteins localized to microtubules. The question arises: why are so many kinds of kinases and phosphatases associated with microtubules? These enzymes might be anchored by microtubules to ensure their exact subcellular localization and prevent their interaction with inappropriate substrates. However, our results with MAPK and the previous work (Ookata *et al.*, 1995; Sontag *et al.*, 1995) suggest that the association is not only for anchoring. Microtubule dynamics are closely related to the phosphorylation of MAPs, which is most likely regulated by the activity of microtubule-associated kinases and phosphatases. The complex phosphorylation states of MAPs may be regulated by the total activity of multiple protein kinases and phosphatases. The equilibrium among these activities might modulate total or local microtubule assembly and stability. Dual specific protein phosphatases (Nebreda, 1994) and PP2A (Sturgill *et al.*, 1988) regulate MAPK negatively. A significant amount of PP2A is associated with microtubules (Sontag *et al.*, 1995). PP2A could work with MAPK cooperatively during mitosis. The activity of both enzymes is regulated reversely in a cell cycle–dependent manner (Gotoh *et al.*, 1991; Sontag *et al.*, 1995). Thus, PP2A might dephosphorylate and inactivate MAPK as well as dephosphorylate the sites phosphorylated by MAPK. MAPK phosphorylation sites on tau are easily dephosphorylated by PP2A (Goedert *et al.*, 1992). Another kinase that is suggested to work with PP2A cooperatively is cdc2/cyclin B, which is also associated with microtubules (Rattner *et al.*, 1990; Tombes *et al.*, 1991) and regulated in the same manner as MAPK during mitosis (Nurse, 1990).

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