Phosphorylation of Microtubule-associated Proteins by a Ca^{2+}/Calmodulin-dependent Protein Kinase

HOWARD SCHULMAN
Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT In an earlier study I demonstrated that rat brain cytosol contains a Ca^{2+}/calmodulin-dependent protein kinase activity that phosphorylates microtubule-associated protein 2 (MAP-2) but not MAP-1. Comparison of sites of phosphate incorporated in MAP-2 catalyzed by the Ca^{2+}/calmodulin-dependent kinase activity and the cyclic AMP-dependent protein kinase activity in cytosolic extracts revealed distinct sites of phosphorylation (Schulman, H., 1984, *Mol. Cell. Biol.*, 4:1175–1178; abstract by me and J. A. Kuret and K. H. Spitzer [1983, *Fed. Proc.*, 42:2250]. I have now used MAP-2 as a substrate to purify the Ca^{2+}/calmodulin-dependent protein kinase responsible for MAP-2 phosphorylation. The brain appears to contain a single predominant Ca^{2+}/calmodulin-dependent protein kinase that phosphorylates MAP-2. The enzyme was purified to apparent homogeneity by column chromatography using DEAE-cellulose, phosphocellulose, hydroxylapatite, Sepharose 6B, and a calmodulin–Sepharose affinity column. The 580,000-dalton holoenzyme consists of 51,000- and 60,000-dalton subunits. The purified enzyme phosphorylates MAP-2 at the same "sites" that are phosphorylated in cytosolic extracts and thus has the same specificity as the activity present in cytosol. Analysis of phosphorylated MAP-2.1 and MAP-2.2, the two components of MAP-2, suggests considerable homology in their phosphorylated domains.

Biochemical and immunocytochemical studies on cytoplasmic microtubules have demonstrated that along with tubulin, the major structural component, there exists numerous microtubule-associated proteins (5, 14, 18, 57, 60, 71). Their designation as such is based on persistent association with microtubules through cycles of assembly–disassembly, immunocytochemical demonstration of co-localization with microtubules, or disruption of their association by antimicrotubule drugs. In brain, a rich source of microtubules, several major microtubule-associated proteins (MAPs)[1] have been identified and partially characterized. These proteins include MAP-1 (59, 67, 68), which is a complex of at least two high molecular polypeptides (350,000 mol wt) and two lower molecular components (28,000 and 30,000 mol wt), MAP-2 (22, 33, 59, 67), which is a 280,000-mol-wt doublet, (MAP-2.1 and MAP-2.2), τ-factor (9, 71), which is composed of four major proteins ranging in size from 55,000 to 70,000 mol wt and several other proteins. In vitro, these proteins decrease the critical concentration of tubulin required for assembly, increase the rate and extent of assembly, and increase the association of microtubules with microfilaments, neurofilaments, and other cellular constituents (15, 23, 25, 28, 38, 40, 44, 45, 52, 56, 58, 61). Efforts at understanding the regulation of microtubule function have focused on the modulatory role of these MAPs.

A high amount of neuronal cyclic AMP(cAMP)-dependent protein kinase is complexed with MAP-2 and is therefore also a MAP (62). This enzyme selectively phosphorylates MAP-2 in preparations of microtubules (59). Extensive studies have demonstrated that MAP-2 is phosphorylated at numerous sites by the cAMP-dependent protein kinase (4, 56, 63). MAP-2 forms periodic projections along the length of microtubules (25, 33, 69). The portion of the molecule closely associated with tubulin and the portion that forms the visible projection are both phosphorylated (66). Other protein kinases phosphorylate MAP-2 as well in that only 10–13 of 20–22 possible phosphates on MAP-2 can be ascribed to the cAMP-dependent protein kinase (63). The effect of MAP-2 phosphorylation on its function in vivo has not been demonstrated. However, several parameters of MAP-2 behavior in vitro are modified by phosphorylation. cAMP-dependent phosphorylation of MAP-2 promotes disassembly, reduces the rate and extent of microtubule assembly, and reduces interaction of microtu-

[1] *Abbreviations used in this paper*: MAP, microtubule-associated protein; PMSF, phenylmethylsulfonyl fluoride.

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bules with actin filaments (28, 40, 56). Similarly, phosphorylation of τ-proteins reduces its association with actin (56).

The potential importance of MAP-2 interactions with microtubules, microfilaments, and intermediate filaments suggests that a study of its regulation by phosphorylation would provide insight into the function of these cytoskeletal elements. In this regard it was of interest to determine whether protein kinases other than the cAMP-dependent protein kinase can regulate its phosphorylation. In view of the importance of Ca^{2+} to microtubule function (12) and the possibility that interaction of MAP-2 with other cellular components may be regulated by Ca^{2+}-dependent phosphorylation, I decided to investigate Ca^{2+}-dependent phosphorylation of MAP-2 in the brain. I have shown that MAP-2 is one of the major, if not the major substrate, for both cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases in rat brain cytosolic extracts and that the "sites" phosphorylated by these two kinases are distinct (see footnote 2; 55). MAP-2 was used as a substrate in order to specifically purify the Ca^{2+}/calmodulin-dependent protein kinase or kinases responsible for its phosphorylation in brain. I report here that a single predominant Ca^{2+}/calmodulin-dependent protein kinase in the brain accounts for all of the Ca^{2+}-dependent phosphorylation of MAP-2 seen in extracts. The enzyme was purified and characterized. Both MAP-2.1 and MAP-2.2, the 280,000-mol-wt subunits of MAP-2, are phosphorylated in cytosol and with purified kinase and MAP-2.

MATERIALS AND METHODS

Materials

Molecular weight markers thyroglobulin, catalase, and aldolase and the Sepharose 4B and 6B resins were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories Inc., Elkhart, IN. Calmodulin was prepared as described (54). DEAE-cellulose and phosphocellulose (P11) were purchased from Whatman Chemical Separation Inc., Clifton, NJ. Hydroxylapatite and SDS electrophoresis reagents (other than acrylamide) were from Bio-Rad Laboratories, Richmond, CA. *S. aureus* cells were from Calbiochem-Behring Corp., San Diego, CA. [γ-^{32}P]ATP (2,000 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL, and all other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Protein Phosphorylation

Male Sprague-Dawley rats (120–180 g) were decapitated and the brain (less cerebellum) was used for preparation of cytosol for endogenous phosphorylation. Brains were homogenized in 3 vol of 25 mM PIPES (pH 7.0), 5 mM EGTA, and 400 μM phenylmethylsulfonyl fluoride (PMSF) with 12 strokes in a glass-Teflon homogenizer at 12,000 rpm at 4°C. Cytosolic extract (13 mg/ml) was obtained by centrifugation at 150,000 *g* for 60 min. The standard assay of endogenous MAP-2 phosphorylation (final volume, 100 μl) contained: 50 mM PIPES (pH 7.0), 5 mM $MgCl_2$, 0.2 mM EGTA (minus Ca^{2+} and minus cAMP), 0.2 mM EGTA + 0.5 mM $CaCl_2$ + 0.5 μg calmodulin (plus Ca^{2+}), 10 μM cAMP + 1 mM 3-isobutyl-1-methylxanthine (plus cAMP), 20 μM [γ-^{32}P]-ATP (2.5 C/mmol), and 65 μg of cytosol protein. MAP-2 kinase activity in column fractions was assayed similarly but with addition of 10 μg of heat-treated microtubules as substrate and 0.1–50 μg of protein from column fractions as kinase. For assay of the cytosolic extract used in purification, additional Ca^{2+} was added to assure the presence of at least 50 μM free Ca^{2+}. After preincubation for 30 s at 30°C the reaction was initiated by addition of [γ-^{32}P]ATP. Incubation was carried out for 1–5 min, terminated by addition of 50 μl of an "SDS-stop solution," heated for 2 min at 100°C, and half the sample was analyzed by SDS PAGE (9 or 6% acrylamide as indicated) and autoradiography as described (47, 64).

[2] Schulman, H. 1984. Differential phosphorylation of MAP-2 stimulated by calcium-calmodulin and cyclic AMP. *Mol. Cell. Biol.* 4:1175–1178.

Molecular weights on SDS gels were calibrated using phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), trypsinogen (M_r 25,000), β-lactoglobin (M_r 18,400), and lysozyme (M_r 14,300) as standards. Protein was visualized by Coomassie Brilliant Blue R-250. Autoradiography was performed with Kodak BB-1 film (Eastman Kodak Co., Rochester, NY) either with or without a Coronex Lightning Plus intensifying screen (Dupont Instruments, Wilmington, DE). Radioactivity was quantitated either by densitometry of the autoradiograph or by measuring Cerenkov radiation in a Packard TriCarb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL).

Preparation of Microtubules and MAP

Authentic bovine brain microtubules were isolated without glycerol by a modification of the procedure of Asnes and Wilson (1) and generously provided by Dr. L. Wilson. Three cycles of assembly and disassembly and a final centrifugation through a 50% sucrose cushion were used to purify the microtubules. MAP-2 and τ-proteins were prepared by fractionation of dissociated microtubules on phosphocellulose (71) followed by a heat treatment at 70°C (see below). Heat treatment of cytosol and microtubules was used to inactivate endogenous kinase and phosphatase activity. Cytosolic extracts or a 1 mg/ml solution of bovine brain microtubules (in 25 mM PIPES, pH 7.0, 1 mM EDTA) were heated at 70°C for 5 min. Such heat treatment completely eliminated endogenous phosphorylation without affecting the ability of MAP-2 to be phosphorylated (see Fig. 5). Such a heat-treated microtubule preparation was used as the MAP-2 substrate for purification of the MAP-2 kinase.

Partial Proteolysis

Labeled MAP-2 was located on stained SDS gels by autoradiography. Gel pieces corresponding to the MAP-2.1 and/or MAP-2.2 were excised from the gel and rehydrated in 1 ml of 62.5 mM Tris (pH 6.7), 0.1% SDS, 1 mM EDTA, and 2.5% 2-mercaptoethanol (vol/vol). After 1 h the buffer was removed and gel pieces, in a glass tube, were heated to 100°C for 3 min. This treatment facilitated extraction of MAP-2 during the proteolysis step. ~95% of ^{32}P-MAP-2 was extracted from the original gel piece during partial proteolysis. Partial proteolysis was performed using 2–5 μg of *S. aureus* V8 protease per sample in 15% SDS polyacrylamide gels (8).

Immunoprecipitation

Endogenous phosphorylation stimulated by either Ca^{2+} or cAMP was carried out as described. The reaction was terminated by chilling to 4°C and making the reaction 0.1% SDS. After 5 min, half of the reaction mixture was immunoprecipitated (48) using a 1/100 dilution of monoclonal antibody to MAP-2 (D-1D1.16.1) (27) kindly provided by Dr. J. Richard McIntosh and Dr. Jonathan G. Izant. The other half of the sample was untreated, kept on ice for the duration of the immunoprecipitation, and used as the nonimmunoprecipitated control. Both control and immunoprecipitated samples were resolved on SDS gels using 6% acrylamide.

Purification of MAP-2 Kinase

In preliminary studies it was apparent that variable elution profiles of the kinase were obtained on DEAE-cellulose chromatography if Ca^{2+} was present, probably due to a variable degree of association of kinase molecules with the highly acidic calmodulin present at this early stage of purification. To prevent association of the kinase with calmodulin and to reduce degradation of the enzyme by Ca^{2+}-dependent protease, the homogenization and subsequent steps in purification were carried out in buffers containing EGTA. The following buffers were used in the purification. All procedures were carried out at 4°C. Buffer A contained 25 mM PIPES (pH 7.0), 5 mM EGTA, 400 μM PMSF, and 1 μg/ml aprotinin; buffer B contained 25 mM PIPES (pH 7.0), 2 mM EGTA, 200 μM PMSF, 1 μg/ml aprotinin, and 10% glycerol; buffer C contained 100 mM $NaKPO_4$ (pH 7.0), 2 mM EGTA, 2 mM 2-mercaptoethanol, 200 μM PMSF, and 10% glycerol; buffer D contained 25 mM PIPES (pH 7.0), 0.5 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 200 μM PMSF, and 10% glycerol; buffer E contained 25 mM PIPES (pH 7.0), 0.2 M NaCl, 1.0 mM EGTA, 2 mM 2-mercaptoethanol, 200 μM PMSF, and 10% glycerol.

PREPARATION OF CYTOSOLIC EXTRACT: In a typical purification, 35 rats (male, Sprague Dawley) were decapitated and brains (less cerebellum) were removed and homogenized by 12 up and down strokes at 900 rpm in a Teflon-glass homogenizer with 3 vol (vol/wt) of buffer A. The homogenate was centrifuged at 900 *g* for 15 min in a Sorvall GSA rotor (DuPont Sorvall, Newtown, CT). The pellet was reextracted with 3 vol of buffer A (same volume as above) and recentrifuged as above. The supernatants were combined and

centrifuged at 50,000 *g* for 60 min in a Sorvall type 19 rotor. The supernatant was made 10% (wt/vol) in glycerol and employed as the cytosolic extract.

DEAE-CELLULOSE FRACTIONATION: The cytosolic extract was applied to a DEAE-cellulose column (2.75 × 34 cm) equilibrated with buffer B and rinsed with 1 bed vol of buffer B. The kinase was eluted from the column with a steep 800-ml linear gradient of NaCl (0–0.8 M) in buffer B. The DEAE-cellulose column fractions containing MAP-2 kinase activity (0.07–0.125 M NaCl) were pooled.

PHOSPHOCELLULOSE FRACTIONATION: The DEAE-cellulose pool was loaded onto a phosphocellulose column (2.75 × 18.5 cm) equilibrated with buffer B containing 0.1 M NaCl. The column was rinsed with 1 bed vol of buffer B and the enzyme was eluted by a 1-liter linear gradient of NaCl (0.1–1.0 M) in buffer B. MAP-2 kinase activity was eluted from the phosphocellulose column between 0.28 and 0.38 M NaCl.

HYDROXYLAPATITE FRACTIONATION: The phosphocellulose pool was applied directly to a hydroxylapatite column (1.42 × 7.5 cm) equilibrated in buffer C. The column was rinsed with 1 vol of buffer C containing 0.5 M NaCl; then the kinase was eluted with a 120-ml linear gradient of $NaKPO_4$ (0.1 M–0.6 M) in buffer C. Active fractions from the hydroxylapatite column (0.17–0.29 M $NaKPO_4$) were pooled.

SEPHAROSE 6B FRACTIONATION: The hydroxylapatite pool was made 50% in ammonium sulfate by addition of solid ammonium sulfate. After 30 min at 4°C, precipitated material was collected by centrifugation at 20,000 *g* for 20 min in a Sorvall SS-34 rotor. The pellet was resuspended in buffer B containing 0.2 M NaCl and applied to a Sepharose 6B column (1.6 × 81 cm) equilibrated in the same buffer. The kinase was eluted as a symmetrical peak between 93 and 105 ml and pooled.

CALMODULIN-SEPHAROSE FRACTIONATION: The Sepharose 6B pool was made 1 mM free Ca^{2+}, just before application on the affinity column. A calmodulin–Sepharose 4B column was prepared by reacting calmodulin with CNBr-activated Sepharose 4B as described by Klee and Krinks (35). The kinase pool was applied to the calmodulin–Sepharose 4B column (0.7 × 8 cm) equilibrated in buffer D containing 0.2 M NaCl. The column was rinsed with 6 bed vol of buffer D containing 0.5 M NaCl and the kinase eluted by step elution with buffer E. The four 1-ml fractions with high MAP-2 kinase activity were pooled and stored at −70°C.

RESULTS

Purification of Ca^{2+}/Calmodulin-dependent MAP-2 Kinase

A representative scheme for the purification of the Ca^{2+}/calmodulin-dependent MAP-2 kinase is described in Materials and Methods. The enzyme was relatively unstable postmortem before preparation of the cytosolic extracts. In a typical purification of 30–50 rat brains, homogenization was performed within 1 min of decapitation. Although Ca^{2+}/calmodulin-dependent kinase activity can be detected in brain using casein or phosvitin as an exogenous substrate, the more cumbersome assay with MAP-2 as substrate, and analysis by SDS gels, was used to specifically purify any and all Ca^{2+}/calmodulin-dependent MAP-2 kinases.

The summary of the purification detailed in the Materials and Methods is presented in Table I. The Ca^{2+}/calmodulin-dependent protein kinase was purified 373-fold from cytosolic extract with a 6% yield. There is some uncertainty in the precise degree of purification required to achieve homogeneity because measurement of MAP-2 kinase activity in crude cytosol is affected by endogenous phosphatases and kinase inhibitors. In several purifications ~0.5 mg of enzyme was obtained from 40 rat brains. The purified enzyme was stable for several months and stable to freeze-thawing if stored in buffer E containing 10–20% glycerol.

Properties of MAP-2 Kinase

The Ca^{2+}/calmodulin-dependent MAP-2 kinase was purified to apparent homogeneity. Analysis of the calmodulin–Sepharose 4B pool by SDS PAGE revealed the presence of a major protein band at 51,000 mol wt and a less prominent doublet at 60,000 mol wt (Fig. 1). Various pieces of evidence suggest that these polypeptides are components of a multisubunit enzyme. These polypeptides co-purified from the hydroxylapatite to the calmodulin–Sepharose step of the purification as a unit in an approximately constant ratio. Attempts to separate these polypeptides under nondenaturing conditions have been unsuccessful. Extensive biochemical analysis of the kinase suggests that the 51,000-mol-wt band and the 60,000-mol-wt doublet are each calmodulin-binding proteins, have binding sites for ATP, and show structural homology (Kuret, J., and H. Schulman, manuscript submitted for publication).

It became apparent during the purification that the kinase undergoes autophosphorylation in the presence of Ca^{2+} and calmodulin. Each of the polypeptides incorporates ^{32}P under conditions of autophosphorylation. Comparison of ^{32}P incorporation into the 51,000- and 60,000-mol-wt subunits indi-

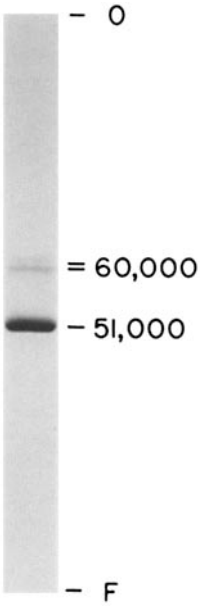

FIGURE 1 SDS PAGE of purified Ca^{2+}/calmodulin-dependent protein kinase. An aliquot (5 μg protein) of the calmodulin–Sepharose pool was analyzed by SDS PAGE (9% acrylamide) as described in Materials and Methods. Protein, stained with Coomassie Brilliant Blue is shown, with molecular weights of subunits indicated. The top of the gel is indicated by *O* and the position of the dye front (bromphenol blue) with *F*.

TABLE I

Purification of MAP-2 Kinase

Fraction	Total Protein	Specific activity	Total activity	Yield	Fold purification
	mg	*nmol/min per mg*	*nmol/min*	%	
Cytosolic extract	2,625	0.074	194	100	1.0
DEAE-cellulose	407	0.606	246	127	8.2
Phosphocellulose	38	3.44	131	67	46
Hydroxylapatite	6.1	6.18	37.8	20	83
Sepharose 6B	1.2	15.3	18.7	9.6	207
Calmodulin–Sepharose	0.42	27.6	11.5	5.9	373

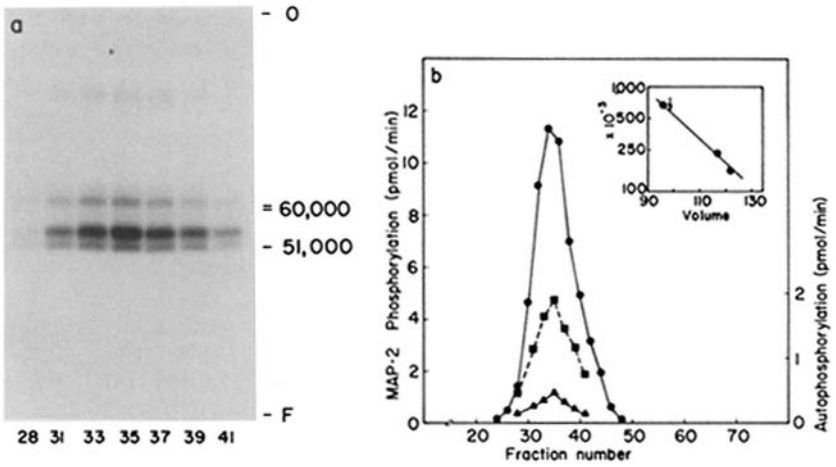

FIGURE 2 Elution of MAP-2 kinase activity and autophosphorylated kinase on Sepharose 6B. Gel filtration on Sepharose 6B was carried out as described in Materials and Methods. Aliquots (10 μl) were assayed either for MAP-2 kinase activity or separately, in the absence of microtubules, for autophosphorylation of the kinase as described in Materials and Methods. (a) Autoradiograph showing autophosphorylation of the 51,000-mol-wt polypeptide and the 60,000-mol-wt doublet in fractions 28–41. Position of unphosphorylated kinase subunits is indicated with their molecular weights. (*b*) MAP-2 kinase activity in the Sepharose 6B fractions was quantitated by Cerenkov radiation (●). ^{32}P incorporation into the kinase subunits, the 51,000-mol-wt band (■) and 60,000-mol-wt doublet (▲) was quantitated by counting appropriate bands from the gel shown above. *Inset* shows elution of MAP-2 kinase relative to protein standards: porcine thyroglobulin (M_r 669,000); bovine catalase (M_r 232,000); aldolase (M_r 158,000). Arrow indicates position of activity peak.

cates that they co-purify from the phosphocellulose step to the homogeneous preparation. The analysis of the Sepharose 6B step is shown in Fig. 2. Column fractions were assayed for endogenous phosphorylation in the presence of Ca^{2+} and calmodulin as described in Materials and Methods. Analysis of phosphoproteins in fractions 28–41 by SDS gels and autoradiography is shown in Fig. 2*a*. Multiple phosphorylation of the 51,000-mol-wt subunit reduces its mobility in SDS gels giving rise to a doublet consisting of a 51,000- and a 53,000-mol-wt band. Phosphorylation of the 60,000-mol-wt doublet also gives rise to a doublet of ^{32}P which is less well resolved but also appears to have reduced mobility. The quantitation of ^{32}P incorporation into the 51,000-mol-wt subunit and the 60,000-mol-wt doublet is shown in Fig. 2*b*. These phosphoproteins co-migrate on Sepharose 6B at a constant ratio to each other and to the MAP-2 kinase activity in these same fractions. MAP-2 kinase activity was eluted as a symmetrical peak centering at 98 ml (K_{av} = 0.60), corresponding to a holoenzyme with a molecular weight of 580,000. Densitometric scans of both protein staining and autophosphorylation from several preparations of the enzyme suggest an ~4:1 ratio of the 51,000-mol-wt subunit to the 60,000-mol-wt doublet, although a precise subunit composition must await more extensive biochemical and biophysical analysis.

Activation of MAP-2 Kinase

The soluble MAP-2 kinase was activated by Ca^{2+} and calmodulin in a manner similar to that seen earlier with the membrane-bound Ca^{2+}/calmodulin-dependent protein kinase from rat brain, the soluble bovine brain enzyme, and several other calmodulin-dependent enzymes (46, 54, 70). Enzymatic activity was absolutely dependent on the presence of both Ca^{2+} and calmodulin. In the presence of Ca^{2+} and calmodulin, phosphorylation of MAP-2 proceeded linearly for 2–3 min and reached a maximal level by 10 min. In the absence of either Ca^{2+} or calmodulin or of both, ^{32}P incorporation into MAP-2 is hardly visible even in deliberately overexposed autoradiographs (Fig. 3*a*, lanes *1–3*). In the presence of both Ca^{2+} and calmodulin, incorporation of ^{32}P into MAP-2, high molecular weight proteolytic fragments of MAP-2 as well as the τ-proteins is greatly increased (Fig. 3*a*, lane *4*). Ca^{2+} and calmodulin stimulate MAP-2 phosphorylation nearly 200-fold above the level seen with either agent alone. The τ-proteins, consisting of four closely related, heat-stable microtubule-associated proteins also promote microtubule assembly (9, 21, 25). They are also very good substrates for the MAP-2 kinase (manuscript in preparation). They are phosphorylated equally well in heat-treated and non-heat-treated preparations of microtubules. By contrast, both α- and β-subunits of tubulin are relatively poor substrates (unpublished). The effect of increasing concentration of Ca^{2+} in the presence of calmodulin is shown in Fig. 3*b*. Ca^{2+} stimulates MAP-2 kinase with a half-maximal rate of phosphorylation occurring at 0.94 μM free Ca^{2+}. The effect of calmodulin in the presence of excess Ca^{2+} is shown in Fig. 3*c*. Half-maximal phosphorylation of MAP-2 occurs at 0.49 μg/ml or 29 nM calmodulin.

MAP-2 was one of the best substrates found for this kinase, with a V_{max} of 225 nmol/min per mg and an apparent K_m of 0.15 μM. The K_m determined using this Ca^{2+}/calmodulin-dependent protein kinase is considerably lower than the K_m of 2.4 μM determined for the cAMP-dependent protein kinase that is associated with MAP-2 (62) and is lower than the concentration of MAP-2 in rat brain (65).

Phosphorylation of MAP-2

Whereas the identity of the cAMP-dependent protein kinase regulating MAP-2 phosphorylation is known, the identity and number of protein kinases responsible for the Ca^{2+}-dependent phosphorylation in rat brain cytosol is unknown. During the purification of the kinase described above, only a single prominent Ca^{2+}-dependent MAP-2 kinase was detected, suggesting that only a single enzyme was involved. In that each protein kinase has its own unique substrate specificity, I

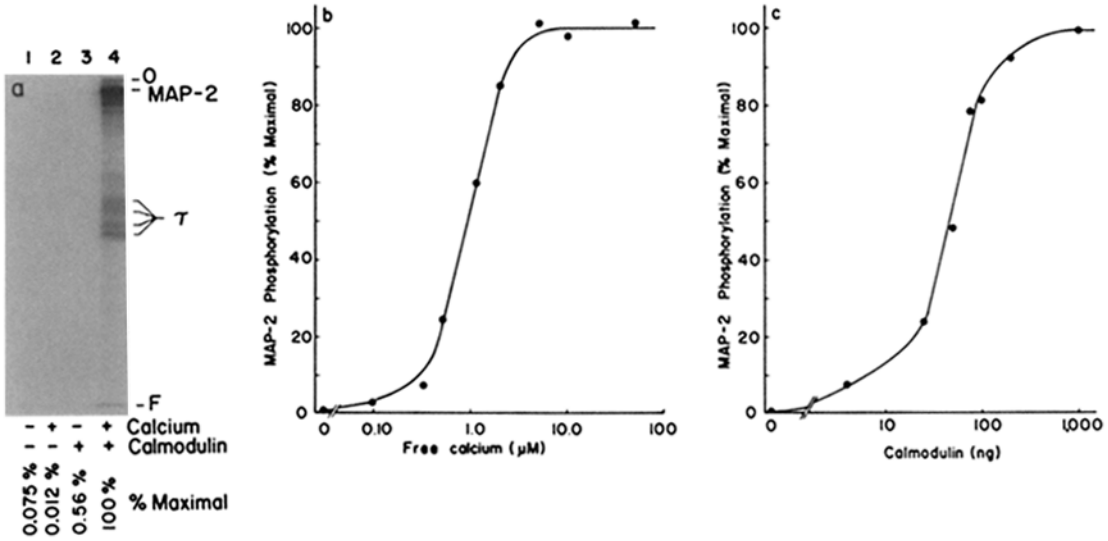

FIGURE 3 Regulation of MAP-2 and τ-protein phosphorylation by Ca^{2+} and calmodulin. (a) MAP consisting of MAP-2 and τ-protein (2 μg protein) was phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase (100 ng) for 1 min under standard conditions in the presence or absence of 0.3 mM free Ca^{2+} and 0.5 μg calmodulin as indicated. Reaction mixtures were resolved on SDS gels (9% acrylamide) and phosphorylated proteins analyzed by autoradiography. A long exposure was used to visualize low level of MAP-2 phosphorylation in lanes *1–3*. The positions of MAP-2 and the four τ-proteins are indicated. ^{32}P incorporation into MAP-2 in the presence of Ca^{2+} and calmodulin (lane *4*) was 25.4 nmol/min per mg. ^{32}P incorporation is given for each lane as percent of maximal levels. (*b*) The MAPs were phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase as above, in the presence of 0.5 μg calmodulin and increasing concentration of free Ca^{2+} as indicated. Free Ca^{2+} concentration was determined by using an apparent binding constant for Ca^{2+}-EGTA of 7.61×10^6 M^{-1} (49). The ^{32}P-labeled band corresponding to MAP-2 was excised from the gel and ^{32}P incorporation was quantitated. Maximal phosphorylation (100%) represents 24.3 nmol of ^{32}P transferred/min per mg. (*c*) MAPs were phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase for 1 min in the presence of 0.3 mM free Ca^{2+} and increasing concentrations of calmodulin as indicated. ^{32}P incorporation into MAP-2 was quantitated as above, with 100% phosphorylation representing 24.0 nmol of ^{32}P transferred/min per mg.

decided to analyze MAP-2 labeled either in cytosolic extracts or by the purified kinase and determine whether the cytosolic enzyme(s) and purified enzyme have similar phosphorylation site specificities.

As a prerequisite for the analysis of phosphopeptides derived from endogenous phosphorylation of MAP-2 in cytosolic extracts it was necessary to ascertain whether all the ^{32}P in the region of the SDS gel where MAP-2 migrates is indeed on MAP-2. Cytosolic extracts were phosphorylated by the endogenous Ca^{2+}-dependent protein kinase(s) or cAMP-dependent protein kinase and analyzed either directly on SDS gels or first immunoprecipitated with monoclonal antibody to MAP-2 and then analyzed on SDS gels. The results of such an analysis are shown in Fig. 4*a*. In typical experiments ~60% of the ^{32}P-labeled high molecular weight band seen in cytosolic extracts was selectively immunoprecipitated. Phosphopeptides were then analyzed by excising the putative MAP-2 band and subjecting it to partial proteolysis using *S. aureus* V8 protease. The results shown in Fig. 4*b* clearly demonstrate that the phosphopeptides generated from the putative MAP-2 in cytosolic extracts labeled with either the cAMP-dependent or Ca^{2+}-dependent protein kinase are present in the ^{32}P-labeled protein immunoprecipitated with MAP-2 antibodies. As shown earlier, the phosphopeptides generated from cAMP-dependent and Ca^{2+}-dependent phosphorylation of MAP-2 are distinct (see footnote 2; 55). Similar results have been seen with two polyclonal antibodies to porcine (10) and calf brain (3) MAP-2. Thus, essentially all of the ^{32}P-labeled 280,000-mol-wt band is MAP-2.

To compare MAP-2 phosphorylated by the endogenous kinase(s) and purified kinase one has to use the same MAP-2 as substrate. Preparations of MAP-2 containing different levels of covalently linked phosphate would show differences in subsequent phosphorylation. To provide identical conditions in the two phosphorylations, heat-treated cytosolic extract was used as the source of MAP-2 for the purified enzyme. Because MAP-2 is heat stable (15), I felt that a heat treatment of a cytosolic extract would inactivate endogenous kinase and phosphatase activity without affecting the ability of MAP-2 to be phosphorylated. Indeed, when purified bovine brain MAP-2 was heat-treated, phosphorylated by the Ca^{2+}/calmodulin dependent protein kinase, and analyzed by partial proteolysis, the phosphopeptides were indistinguishable from those generated from native MAP-2 that was similarly phosphorylated and analyzed (Fig. 5, lanes 3 and 4). Apparently the ability of MAP-2 to serve as substrate for the Ca^{2+}-dependent kinase is not affected by prior heating. No new sites for phosphorylation are generated and none are eliminated. Similar stability of phosphorylation sites is seen with the cAMP-dependent protein kinase (data not shown). The two forms of ^{32}P–MAP-2 were prepared. First, endogenous phosphorylation in rat brain cytosol was carried out in the presence of Ca^{2+} under conditions that label MAP-2. An equivalent amount of cytosolic extract was then heat-treated and phosphorylated by the purified kinase. The two phosphorylation reactions were resolved on SDS gels, and the partial proteolysis pattern of the MAP-2 bands was examined. As shown in Fig. 5 (lanes *1* and *2*), the phosphopeptide pattern of MAP-2 labeled by the purified Ca^{2+}/calmodulin-dependent protein kinase is indistinguishable from that seen with the endogenous kinase(s) in the cytosolic extract. Inasmuch as only a single Ca^{2+}-dependent MAP-2 kinase was detected

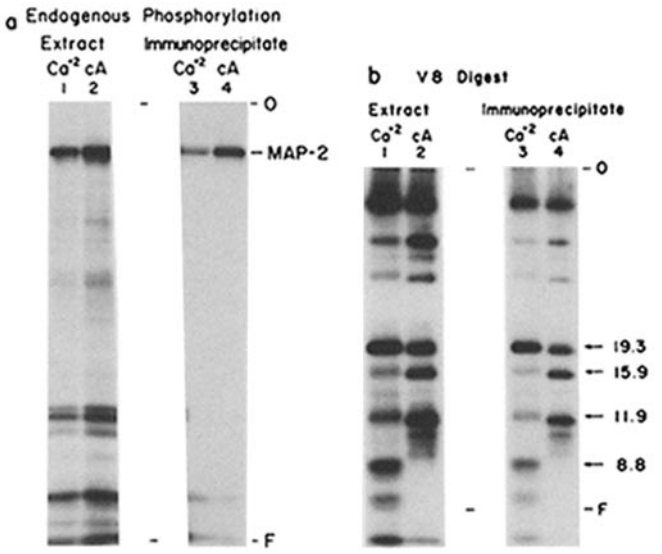

FIGURE 4 Immunoprecipitation and partial proteolysis of MAP-2 from rat brain cytosol. Endogenous phosphorylation in rat brain cytosolic extracts was stimulated by cAMP or Ca^{2+}/calmodulin, samples were immunoprecipitated by a monoclonal antibody to MAP-2 (clone D-1D1.16.1) (27), and were analyzed by SDS gels (6% acrylamide). (*a*) Autoradiograph of cytosolic extract labeled by endogenous cAMP-dependent phosphorylation (*cA*) or Ca^{2+}/calmodulin-dependent phosphorylation (Ca^{2+}) analyzed before immunoprecipitation (lanes *1* and *2*). Autoradiograph of equal aliquots of samples above but after immunoprecipitation (lanes *3* and *4*). (*b*) The putative MAP-2 band of lanes 1–4 (*a*) was excised and subjected to partial proteolysis using *S. aureus* V8 protease as described in Materials and Methods. Lanes *1–4* correspond to phosphorylation conditions in lanes 1–4 of a. The molecular weight of the four major Ca^{2+} phosphorylation-generated phosphopeptides are indicated in kilodaltons. Dye front is that of Pyronin Y used in the proteolysis gels.

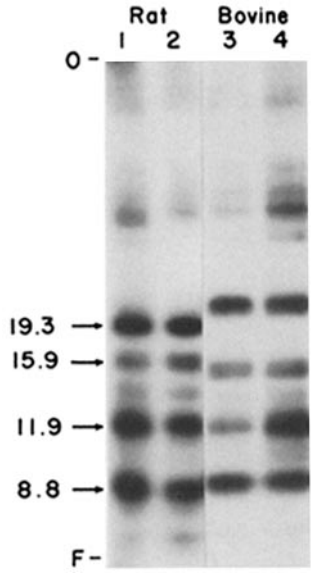

FIGURE 5 Comparison of MAP-2 phosphopeptides from endogenous phosphorylation and from phosphorylation by the purified kinase. A preparation of bovine brain microtubules cycled three times was phosphorylated by the purified Ca^{2+}/calmodulin-dependent protein kinase either with or without prior heat-treatment, as described in Materials and Methods. The ^{32}P-labeled MAP-2 was excised from the SDS gels and partial proteolysis performed as described. Autoradiographs corresponding to bovine MAP-2 phosphorylated in the native (lane *4*) or heat-treated (lane *3*) state are shown above. Endogenous phosphorylation in rat brain cytosol was performed as described. An equal aliquot was first heat-treated and then phosphorylated by addition of 200 ng of purified Ca^{2+}/calmodulin-dependent protein kinase. The ^{32}P-labeled MAP-2 bands from an SDS gel of endogenous phosphorylation (lane *1*) or phosphorylation with purified kinase (lane *2*) were subjected to partial proteolysis as above and autoradiograph shown. Samples of rat MAP-2 and bovine MAP-2 were run on the same proteolysis gel but a longer exposure of rat MAP-2 samples was used to facilitate the comparison. Molecular weights ($\times 10^{-3}$) of rat MAP-2 phosphopeptides are indicated.

during the purification and the phosphorylation sites using endogenous and exogenous kinase are indistinguishable, it would appear that the isolated enzyme is the predominant Ca^{2+}/calmodulin-dependent MAP-2 kinase in rat brain cytosol. It was also noted that the phosphopeptide pattern of bovine and rat MAP-2 are similar but distinct, suggesting some differences in the region of the proteins near the phosphorylation sites. The phosphopeptide pattern of cAMP-dependent kinase-labeled MAP-2 from rat and bovine brain are also similar but distinct (data not shown).

On SDS gels with high resolution, MAP-2 from both rat and bovine brain migrates as a doublet designated as MAP-2.1 and MAP-2.2. The structural relationship between these polypeptides has not been defined. Because both MAP-2.1 and MAP-2.2 are heat stable, it was possible to determine whether both bands were phosphorylated by Ca^{2+}-dependent phosphorylation and whether the endogenous kinase and purified kinase show similar phosphorylation of the two polypeptides. The phosphorylation of MAP-2.1 and MAP-2.2 is shown in Fig. 6. Authentic bovine microtubules were phosphorylated by the purified kinase either with or without heat treatment. The phosphorylated sample was analyzed on SDS gels under conditions that resolve MAP-2.1 from MAP-2.2. The individual bands were excised from the gel and analyzed by partial proteolysis as above (Fig. 6, lanes *5–8*). Bovine MAP-2.1 (odd numbered lanes) and MAP-2.2 (even numbered lanes) are nearly indistinguishable in both heated (Fig. 6, lanes *5* and *6*) and unheated samples (Fig. 6, lanes *7* and *8*). Again, there appears to be little difference in phosphorylation generated by heating. There is some reduction in minor peptides that probably result from cAMP-dependent kinase activity in the unheated sample and which is eliminated by heating.

Endogenous MAP-2 phosphorylation in rat brain cytosolic extract was therefore compared with MAP-2 phosphorylated by the purified enzyme. The results (Fig. 6, lanes *1–4*) indicate that both MAP-2.1 and MAP-2.2 from rat brain are phosphorylated by the Ca^{2+}-dependent protein kinase. From the phosphopeptide pattern it is clear that they bear a striking similarity in the regions of the protein that is phosphorylated. In addition, as shown earlier for total MAP-2, all the sites phosphorylated in cytosolic extract (Fig. 6, lanes *1* and *2*) can be phosphorylated by the purified enzyme (Fig. 6, lanes *3* and *4*), thus providing additional evidence that the phosphorylation seen in cytosol is due to activation of the Ca^{2+}/calmodulin-dependent protein kinase described above.

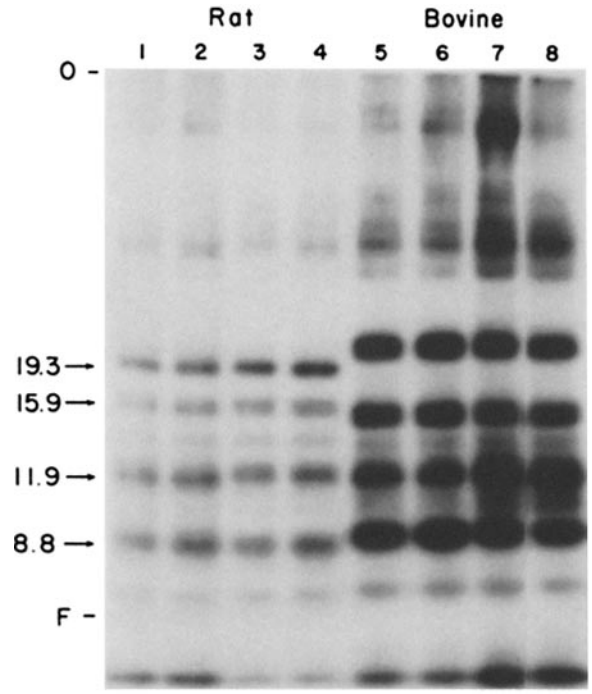

FIGURE 6 Purified bovine microtubules and rat brain cytosolic extract were phosphorylated as in Fig. 5. Samples were analyzed on SDS gels (6% acrylamide) to resolve ^{32}P-labeled MAP-2.1 (all odd-numbered lanes) and MAP-2.2 (all even numbered lanes) and each subjected to partial proteolysis. Autoradiography of rat brain MAP-2.1 and MAP-2.2 with (lanes *3* and *4*) and without (lanes *1* and *2*) prior heat treatment and bovine brain MAP-2.1 and MAP-2.2 with (lanes *5* and *6*) and without (lanes *7* and *8*) heat treatment are shown. The sample in lane *8* was electrophoresed in the same gel apparatus but on a separate gel than lanes *1–7*. Molecular weights (× 10^{-3}) of rat MAP-2 phosphopeptides are indicated.

DISCUSSION

MAP-2 Kinase

Since the initial findings that MAP-2 could be phosphorylated by the cAMP-dependent protein kinase, attention has been focused on the nature and extent of the phosphorylation as well as the possible effects of phosphorylation on MAP-2 function. Recent studies have indicated that bovine MAP-2 contains 8–13 mol phosphate per mol MAP-2 (MAP 2.1 + MAP 2.2) (63). The cAMP-dependent protein kinase can increase this level substantially, up to a maximum of 20–22 mol phosphate (26, 63). Reducing phosphate level by treatment with phosphatases does not generate sites for subsequent incorporation of phosphate catalyzed by the cAMP-dependent protein kinase. Most of the phosphate present in the isolated MAP-2 is therefore not likely to have been added in vivo by the cAMP-dependent protein kinase. It has been suggested that other enzymes possibly including the neurofilament-associated protein kinase (51) may be involved (63).

The most direct approach to identify the enzyme(s) responsible for MAP-2 phosphorylation is to use MAP-2 as a substrate for purification of any and all neuronal kinases capable of phosphorylating MAP-2. The search for the enzyme(s) responsible for the Ca^{2+}-dependent phosphorylation reported here indicates that there is only one predominant Ca^{2+}/calmodulin-dependent protein kinase capable of phosphorylating MAP-2. Only a single peak of activity could be detected from the earliest step in the purification. The enzyme was purified 370-fold from rat brain cytosolic extracts to apparent homogeneity. It thus constitutes a major protein kinase in rat brain, accounting for as much as 0.3% of the soluble protein. Based on gel filtration data it behaves as a 580,000-mol-wt complex consisting of a 51,000-mol-wt polypeptide and a 60,000-mol-wt doublet as subunits. Unlike the cAMP-dependent protein kinase, this enzyme does not appear to form a stable complex with MAP-2. It is not co-purified with microtubules through cycles of assembly–disassembly (unpublished). It is possible, since the enzyme is more temperature labile than the cAMP-dependent protein kinase, that it is inactivated during the 37°C treatments used to induce polymerization. The Ca^{2+}/calmodulin-dependent kinase also differs from the three best characterized second messenger responsive protein kinases the cAMP-, cyclic guanosine monophosphate-, and the Ca^{2+}/phospholipid-dependent protein kinases, in the stringency of its regulation. Each of these shows substantial activity in the absence of its specific activator (19, 34, 50). In the absence of either Ca^{2+} or calmodulin, however, MAP-2 kinase activity is negligible. A minimum of 200-fold stimulation is obtained when the purified enzyme is stimulated by Ca^{2+} plus calmodulin.

A prominent feature of the enzyme is its autophosphorylation. This allows the "visualization" of the 51,000- and 60,000-mol-wt subunits from the first chromatographic step, before it can be detected by protein staining. The co-purification of the autophosphorylated bands in a constant ratio is consistent with their being subunits of the same enzyme. The three polypeptides bind ^{125}I-calmodulin and 8-N_3-ATP independently of each other (Kuret, J., and H. Schulman; submitted for publication), suggesting that each has a calmodulin-stimulated catalytic site capable of a phosphotransferase reaction. Based on partial proteolysis, the 51,000- and 60,000-mol-wt polypeptides share some structural homology at the sites of autophosphorylation. Most protein kinases show some degree of autophosphorylation (e.g., 13, 24, 32, 39). The effect of autophosphorylation on the activity or behavior of the Ca^{2+}-dependent MAP-2 kinase is not known.

The MAP-2 kinase described here has some similarities to and differences from several Ca^{2+}/calmodulin-dependent protein kinases that have recently been described. Four laboratories have purified Ca^{2+}/calmodulin-dependent protein kinases that are claimed to be distinct from each other. These enzymes have been purified using myosin light chains (17), tryptophan hydroxylase (73), synapsin I (2), or tubulin (20) as exogenous substrates, although all appear to have broad substrate specificity and can phosphorylate MAP-2. All preparations except the enzyme purified as a myosin light-chain kinase show a similar subunit composition. The molecular weights of the holoenzymes vary from 540,000 to 650,000. Major differences in substrate specificity have been reported. Synapsin I is an excellent substrate and tubulin a poor substrate for synapsin I kinase (2) whereas the reverse is true of the tubulin kinase (20). It is not yet possible to determine whether these represent the same enzyme that has been prepared and assayed under different conditions or whether, as claimed by the investigators, that they constitute a family of related enzymes. The MAP-2 kinase reported here most clearly resembles the synapsin I kinase. Bovine brain tubulin is not a very good substrate for the enzyme. More extensive

biochemical analysis and direct comparison of the various preparations will be necessary before it is known how many multifunctional Ca^{2+}/calmodulin-dependent protein kinases exist in brain. If, in fact, several related kinases exist, it would be most logical to use the substrate of interest in the purification of the kinase. In the purification of the MAP-2 kinase, the purified enzyme can account for all the phosphorylation seen in the cytosolic extract and is therefore likely to be the major Ca^{2+}/calmodulin-dependent protein kinase phosphorylating MAP-2.

MAP-2 and τ are greatly enriched in rat brain. MAP-2 may account for as much as 1% of total cytoplasmic protein in porcine brain and at 1/100th of this level in non-neuronal porcine tissues (65). Similarly, the level of the Ca^{2+}-dependent kinase is far lower in non-neuronal tissues than in brain (unpublished). It is possible that the kinase is concentrated in brain because one of its major neuronal functions is to regulate MAP-2 and τ-phosphorylation. However, numerous other proteins appear to be excellent substrates for this protein kinase in cytosolic extract as well as with purified enzyme and substrates (see footnote 2; J. Kuret and H. Schulman, manuscript submitted for publication). The use of the term MAP-2 kinase is simply operational. The enzyme appears to have the broad specificity expected of a "general" protein kinase and is thus quite different from other calmodulin-dependent protein kinases such as myosin light-chain kinase and phosphorylase kinase (53). I believe that, by analogy with the cAMP-dependent protein kinase, this enzyme represents a general protein kinase thay may mediate multiple effects of Ca^{2+} in brain and other tissues.

MAP-2

MAP-2 consists of at least two high molecular polypeptides designated MAP 2.1 and MAP 2.2. Most studies to date have dealt with these as a single entity. The relationship between these two proteins is not known. Whether individual MAP-2 molecules consist of both subunits has not been determined. There appear to be species differences in the ratios of MAP 2.1 to MAP 2.2 present in microtubule preparations (unpublished). Both polypeptides are phosphorylated by the purified Ca^{2+}/calmodulin-dependent and cAMP-dependent protein kinases, as well as by endogenous kinases in cytosolic extracts. Analysis of phosphopeptides from MAP-2 labeled by either kinase shows considerable structural homology between MAP-2.1 and MAP-2.2. Inasmuch as each kinase phosphorylates both polypeptides, the differential phosphorylation of MAP-2 by the two kinases reported earlier is not due to their selective phosphorylation of the polypeptides.[2] Recent studies in this laboratory have indicated that the MAP-2 kinase can incorporate more phosphate into MAP-2 than the cAMP-dependent protein kinase and that there is very little overlap in the phosphorylation sites (manuscript in preparation). This enzyme may therefore account for some of the cAMP-independent sites found on MAP-2 (63) as well as new sites previously not recognized. Phosphorylation of MAP-2 at additional sites may add a new dimension to the regulation of MAP-2 function. In fact, in a recent report addition of a Ca^{2+}/calmodulin-dependent kinase similar to the one described here resulted in the disassembly of preassembled microtubules (72). It is therefore possible that changes in the level of either cAMP or Ca^{2+} leads to a change in microtubules mediated by protein phosphorylation.

Several studies on localization of MAP-2 in brain suggest that it is largely confined to dendritic processes and concentrated in dendritic spines (6, 42, 43). The dendritic spines have generated considerable excitement because of their potential role in modifying synaptic efficacy (11). These structures may change their shape and electrical properties in response to stimulation from their presynaptic inputs (16, 37). Dendritic spines in Purkinje cells are densely packed with 4–6- and 5–7-nm filaments of unknown composition and 8–10-min microfilaments that may be actin (36). Microtubules have not been observed but tubulin is concentrated in postsynaptic densities present in the spines (29, 42). Actin has been found to be concentrated in the spines and in association with postsynaptic densities in the spine (6, 41). These filamentous structures may be reponsible for maintenance and control of spine shape. It is intriguing to speculate that MAP-2, which has been shown to interact with tubulin and actin, may be concentrated in synaptic spines for this purpose. Phosphorylation of MAP-2 by the cAMP-dependent protein kinase (62) or the Ca^{2+}/calmodulin-dependent MAP-2 kinase may regulate such interactions involving MAP-2 and thus modulate some of the shape changes. The soluble MAP-2 kinase has properties that are similar to those of the membrane-bound Ca^{2+}/calmodulin-dependent protein kinase reported earlier (54). That enzyme has been shown to be one of the major components of the postsynaptic density, based on ^{125}I-calmodulin binding (7), activity (21), and most recently by phosphopeptide analysis of the 51,000-dalton subunit (30). The relationship between the soluble and membrane-bound activities is not known although they appear quite similar (31). Based on the characteristics of MAP-2 phosphorylation in vitro, the most consistent model of its regulation would involve a reduced interaction of MAP-2 with cytoskeletal elements upon phosphorylation by either the cAMP-dependent or Ca^{2+}/calmodulin-dependent protein kinase. Synaptic activity of some neurotransmitters would elicit changes in cAMP, whereas others would elicit changes in Ca^{2+} and regulate spine function differentially by selective activation of distinct protein kinases that would phosphorylate MAP-2, τ, and other proteins.

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