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Separation of endogenous calmodulin- and cAMP-dependent kinases from microtubule preparations

(tubulin/microtubule-associated protein 2/cytoskeleton/phosphopeptides)

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ABSTRACT **Both cAMP- and calmodulin-dependent kinases are proposed regulators of microtubule function by means of their ability to phosphorylate microtubule-associated protein 2 (MAP 2). A cAMP-dependent kinase/MAP 2 complex is endogenous to microtubules. In this report, we demonstrate that an endogenous calmodulin-dependent kinase that phosphorylates MAP 2 as a major substrate is also present in microtubules prepared under conditions that preserve kinase activity. This enzyme is identical to a calmodulin-dependent kinase purified previously from rat brain cytosol. A fraction containing calmodulin-dependent kinase and MAP 2 was separated from the cAMP-dependent kinase/MAP 2 complex by gel filtration chromatography of microtubule protein in high ionic strength buffer. All of the recovered calmodulin-dependent kinase activity in microtubules eluted in a single protein peak. The specific activity of the enzyme for MAP 2 was enriched 31-fold in this fraction compared to cytosol. Two-dimensional tryptic phosphopeptide mapping revealed that the endogenous cAMP- and calmodulin-dependent kinases phosphorylated distinct sites on MAP 2. These data demonstrate that both kinases are present in microtubule preparations and that they may differentially regulate MAP 2 function by phosphorylating separate sites on MAP 2.**

Microtubules play a pivotal role in a variety of dynamic intracellular processes. *In situ* and *in vitro* studies indicate that microtubules are crosslinked to one another, neurofilaments and other intermediate filaments, actin, and secretory vesicles through the high molecular weight microtubule-associated protein 2 (MAP 2) (for review, see ref. 1). Rapid modulation of cytoskeletal function may be effected by reversible phosphorylation of MAP 2. MAP 2 is phosphorylated *in vivo* (2) and reconstitution studies indicate that phosphorylation of MAP 2 with cAMP-dependent kinase (3) or calmodulin-dependent kinase (4) diminishes its ability to promote tubulin assembly into microtubules. A cAMP-dependent kinase activity is endogenous to microtubule preparations and phosphorylates 8–13 of the 20–22 available sites on MAP 2 (1).

A number of investigations have demonstrated the importance of calmodulin in regulating microtubule dynamics. *In situ* and *in vitro* studies indicate that physiological concentrations of calcium produce rapid disassembly of microtubules when calmodulin is present (5, 6). A subpopulation of microtubules, designated cold-stable microtubules, is exquisitely sensitive to the depolymerizing effects of calcium and calmodulin (7). Microtubules contain calmodulin-binding proteins (8, 9), and calmodulin localizes on microtubules in cultured cells (10, 11). Finally, phosphorylation of MAP 2 with an exogenous brain calmodulin-dependent kinase inhibits its MAP 2-stimulated microtubule assembly (4). These studies suggest that a calmodulin-regulated kinase that phosphorylates MAP 2 may be associated with microtubules and may control microtubule dynamics.

Our laboratory has demonstrated that cytosol preparations contain a calmodulin-dependent kinase activity that phosphorylates MAP 2 and tubulin and we have recently isolated and characterized this enzyme (12, 13). It appears to be a type II calmodulin-dependent kinase (14). We now report that this kinase is present in microtubule preparations, can be separated from the endogenous cAMP-dependent kinase by gel filtration chromatography, and phosphorylates several distinct sites on the MAP 2 molecule.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (5–10 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. ^{125}I-labeled calmodulin (^{125}I-calmodulin) was a gift of F. Gorelick (Department of Cell Biology, Yale University School of Medicine). Molecular weight standards were obtained from Pharmacia. Calmodulin was purified from calf brain by chromatography on DEAE-cellulose (Whatman) and Affi-Gel–fluphenazine (15). Affi-Gel–fluphenazine resin and Bio-Gel A-15m were purchased from Bio-Rad. All other chemicals used in the experiments were reagent grade and were purchased from commercial sources.

Methods. Microtubules were prepared by two cycles of temperature-dependent assembly/disassembly (16) with modifications to preserve kinase activity. Rat brains were rapidly excised and homogenized in <15 sec in ice-cold buffer A [100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)/1 mM EGTA/0.5 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, pH 6.7, 2 ml per brain]. The homogenate was centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant was supplemented with glycerol and GTP to obtain final concentrations of 3.4 M and 1 mM, respectively, and transferred to a shaking water bath for 20 min at 37°C. The incubated mixture was then centrifuged at 75,000 × *g* for 25 min at 37°C. The resultant microtubule pellet was gently resuspended in buffer A (1–4 mg/ml), placed on ice for 20 min to solubilize cold-labile microtubules, and centrifuged at 75,000 × *g* for 25 min at 4°C to obtain a cold-stable microtubule pellet and a supernatant. The supernatant was supplemented with GTP and glycerol as described above, incubated at 37°C, and centrifuged at 75,000 × *g* for 25 min at 37°C to obtain a twice-cycled microtubule pellet. The pellet was then placed on ice to solubilize microtubules and used in

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Abbreviations: MAP 2, microtubule-associated protein 2; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TFP, trifluoperazine.

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the experiments. It is imperative that this protocol be followed in order to preserve calmodulin-dependent kinase activity. Microtubule protein was resolved into two primary protein peaks by gel filtration chromatography as described (17, 18). Depending upon the preparation conditions, the high molecular weight peak of protein contains oligomeric tubulin and associated MAPs, including single or double ring structures that have been designated as 30S (18) or 36S (17) tubulin, respectively. We have confirmed the presence of ring structures in this fraction by electron microscopy, but sedimentation velocity analysis was not performed. This fraction shall be referred to as tubulin rings. The lower molecular weight protein peak contains a large proportion of 6S tubulin (17, 18). Throughout the experimental protocol, the formation or depolymerization of microtubules and tubulin rings was monitored by negative-stain electron microscopy (19).

Calmodulin-dependent kinase activity associated with microtubule protein was assayed as described (12, 13), except that 20 mM Mes (pH 6.7) replaced Pipes as the buffer. In some experiments, the recovery and specific activity of calmodulin-dependent MAP 2 kinase activity in microtubule fractions was followed through repeated cycles of assembly/disassembly (Table 1). Calmodulin-dependent phosphorylation of MAP 2 was calculated as the difference between total MAP 2 phosphorylation in buffer containing magnesium, calcium, and calmodulin [± 0.1 mM trifluoperazine (TFP)]. As described previously, TFP inhibits calmodulin-dependent kinase activity, whereas magnesium-dependent kinase is not affected (12). Inclusion of calcium in the buffer inhibits the endogenous cAMP-dependent kinase activity (Fig. 1). Unless otherwise indicated, samples were incubated for 1 min at 37°C with 7 μM ATP. Microtubule protein was resolved by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE), stained with Coomassie blue or silver, and exposed for autoradiography as described (13, 20, 21). Two-dimensional gel electrophoresis was performed by a modification of the original method (22) as described (13). Phosphorylation of specific substrates was assessed by excision of specific bands from $NaDodSO_4$/PAGE gels and scintillation spectroscopy (20) or by densitometric scanning of gels and autoradiographs (21). Phosphopeptide mapping of microtubule protein was performed as described (13). ^{125}I-Calmodulin binding to polypeptides in denaturing gels was performed as described (13, 23). Protein was determined by the method of Bradford (24) using bovine serum albumin as a standard.

RESULTS

Calmodulin- and cAMP-Dependent Kinase Activities in Microtubule Preparations. Fig. 1 shows the protein pattern and autoradiograph of microtubule protein that was assayed for calmodulin- or cAMP-dependent kinase activity. Calmodulin-dependent incorporation of phosphate was observed in several protein bands at M_r 52,000–63,000, M_r 80,000–86,000, and M_r 280,000, the latter corresponding to MAP 2. In the absence of calmodulin, a magnesium-dependent, calcium-inhibited incorporation of phosphate into MAP 2 was observed. As described previously (2), addition of cAMP stimulated MAP 2 phosphorylation by ≈4-fold. The combined presence of both cAMP- and calmodulin-dependent kinases in microtubule fractions makes it difficult to absolutely assess the activity of each kinase, independent of the other. The calmodulin-dependent kinase activity reported in Table 1 was assessed by the difference between phosphorylation in the presence and absence of TFP.

Table 1 presents the recovery and specific activity measurements of calmodulin-dependent MAP 2 kinase activity in microtubule preparations compared to brain cytosol. Once-cycled microtubules retained ≈78% of the recovered activity

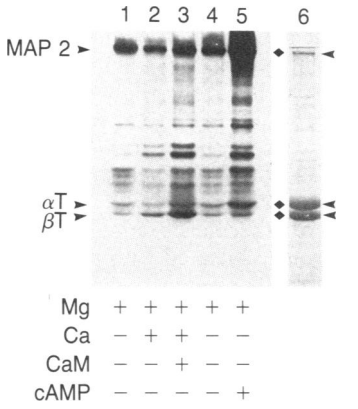

FIG. 1. Calmodulin-dependent and cAMP-dependent kinase activity in microtubules. Protein pattern (lane 6) and autoradiograph (lanes 1–5) of phosphorylated microtubule protein (twice-cycled) resolved by one-dimensional $NaDodSO_4$/PAGE. Protein was incubated in buffer containing the additions indicated: Mg = magnesium, Ca = calcium, CaM = calmodulin, and cAMP = 10 μM cAMP. The positions of MAP 2, α tubulin (αT), and β tubulin (βT) are depicted.

in comparison to cytosol and the calmodulin-dependent MAP 2 kinase activity was enriched by 4-fold. Cold-stable microtubules contained 56% of the enzyme activity present in cytosol and the specific activity of the kinase was enriched by 16-fold. Twice-cycled microtubules contained 14% of calmodulin-dependent kinase activity in cytosol, representing a 3-fold enrichment. The calmodulin-dependent kinase activity in cytosol is significantly inactivated by incubation of microtubule protein at 37°C (9, 25), whereas the amount of enzyme inactivation is less during subsequent incubation of microtubule protein, suggesting that an inactivating factor is removed after centrifugation of the cytosol. There remains a significant amount of enzyme activity in twice-cycled microtubule preparations. Twice-cycled microtubules were employed for further studies since they contain both cAMP- and calmodulin-dependent kinase activities and very little actin and other protein contaminants compared to cold-stable microtubules.

Separation of Endogenous Calmodulin- and cAMP-Dependent Kinase Activities in Microtubules. Depolymerized microtubule protein was resolved into tubulin rings and 6S tubulin by gel filtration chromatography (17, 18). Unlike 6S tubulin, tubulin rings are competent for repolymerization into microtubules due to the presence of MAP 2 in the ring (18). The cAMP-dependent kinase activity coelutes with the rings since it is tightly bound to the MAP 2 molecule (ref. 1; Fig.

Table 1. Recovery and specific activity of MAP 2 calmodulin-dependent kinase activity in microtubule preparations

Preparation	Recovery, %	Specific activity, pmol of PO_4 per min/mg of protein
Cytosol	100	7.6 (1)
Once-cycled	78	33.6 (4)
Cold-stable	56	118.4 (16)
Twice-cycled	14	22.6 (3)
Void fraction	5	234.0 (31)

Cytosol or microtubule protein was incubated under standard phosphorylation conditions and subsequently resolved on one-dimensional $NaDodSO_4$/PAGE. Protein with M_r 280,000 (MAP 2) was excised, and incorporated radioactive phosphate was quantitated by liquid scintillation spectroscopy. Calmodulin-dependent MAP 2 kinase activity in microtubule fractions was compared to enzyme activity in cytosol. All samples were supplemented with added MAP 2 so that substrate was not rate-limiting. Fold enrichment is shown in parenthesis.

2*A*). Fig. 2*A* demonstrates that all of the calmodulin-dependent kinase activity also coelutes with the tubulin rings near the void volume of the column. Autoradiographs of representative samples from the void, trough, and back protein peaks are shown in Fig. 3*A*. As described previously, protein from the front peak formed microtubules when incubated with GTP at 37°C, whereas 6S tubulin did not form microtubules (17, 18).

To determine whether the observed calmodulin-dependent kinase activity was associated with tubulin rings or another coeluting high molecular weight complex, NaCl was added to the microtubule protein prior to gel filtration chromatography. This treatment has been shown to disassemble the rings into a cAMP-dependent kinase/MAP 2 complex, MAP 2, and 6S tubulin (1, 17, 18). Fig. 2*B* shows the Bio-Gel A-15m elution profile for protein and calmodulin- and cAMP-dependent kinase activities in NaCl-treated microtubules. There was a significant reduction in the amount of protein in the void peak from the column, which is consistent with the dissolution of rings into lower molecular weight species. However, all of the recovered calmodulin-dependent MAP 2 kinase activity still eluted in the void fraction, representing an enzyme enrichment of 10-fold over twice-cycled microtubule protein. Approximately 10% of the MAP 2 applied to the column eluted in the void fraction. Endogenous Mg/cAMP-dependent MAP 2 kinase activity was no longer seen in the void peak, although some of this kinase may be present in an inactivated form (Fig. 3*B*). Approximately 80% of the MAP 2 applied to the column was recovered in between the two main peaks of protein (trough), and the Mg/cAMP-stimulated phosphorylation of MAP 2 was shifted to this lower molecular weight trough fraction (fraction numbers 26–40). This observation is consistent with the recovery of a cAMP-dependent kinase/MAP 2 complex from NaCl-treated, chromatographed microtubule protein (1). Therefore, two

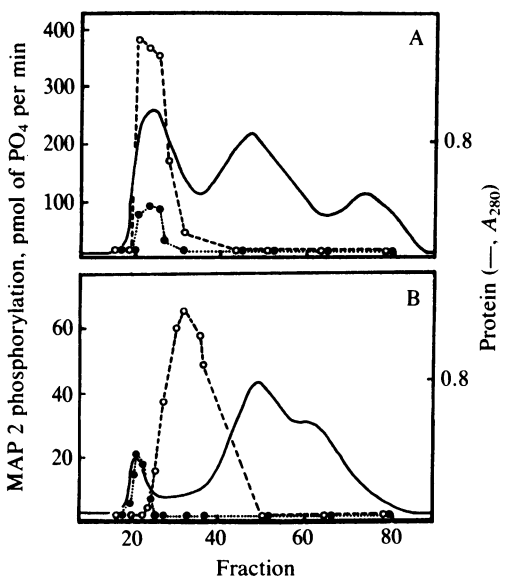

FIG. 2. Separation of calmodulin-dependent and cAMP-dependent kinase activities in microtubules. (*A*) Without NaCl. Twice-cycled microtubule protein was chromatographed on Bio-Gel A-15m and phosphorylated under standard conditions, and fractions were assayed for calmodulin-dependent MAP 2 phosphorylation (●···●) and cAMP-dependent MAP 2 phosphorylation (○----○). The ordinate values represent the total kinase activity in each corresponding fraction and were obtained by multiplying the kinase activity in an aliquot from the fraction by the total fraction volume. The elution of protein was monitored at 280 nm (—). Column buffer contained 10 mM Mes and 0.1 mM $MgCl_2$ (pH 6.75). (*B*) Experimental conditions were as described in *A*, except that 500 mM NaCl was added to microtubule protein and column buffer.

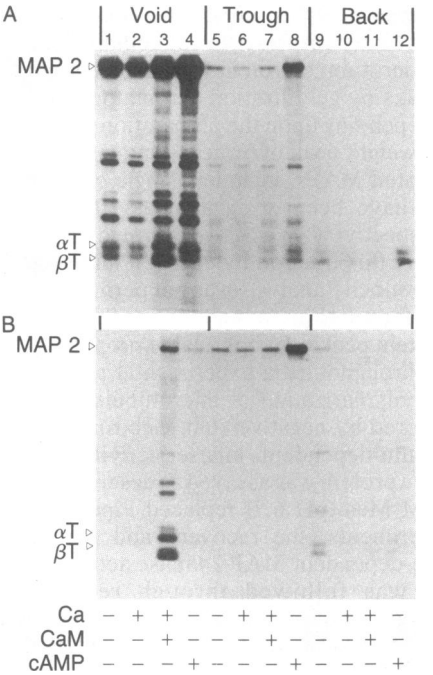

FIG. 3. Phosphorylation of microtubule protein by endogenous calmodulin-dependent and cAMP-dependent kinase activities in microtubules. (*A*) Autoradiograph of microtubule protein corresponding to samples from the profile shown in Fig. 2*A*. Void = fraction 24, Trough = fraction 32, and Back = fraction 44. (*B*) Autoradiograph of microtubule protein corresponding to samples from the profile shown in Fig. 2*B*. Void = fraction 22, Trough = fraction 34, and Back = fraction 52. All samples (*A* and *B*) were phosphorylated under standard conditions in buffer containing the additions indicated and subsequently resolved on one-dimensional $NaDodSO_4$/PAGE (7% acrylamide). Magnesium was present in all samples.

distinct MAP 2 fractions containing either cAMP- or calmodulin-dependent kinase were prepared.

Identification of Calmodulin-Dependent Kinase in Microtubule Preparations. Studies were conducted to characterize the endogenous, microtubule-associated calmodulin-dependent kinase that was separated from endogenous cAMP-dependent kinase. The calmodulin-dependent kinase in the void fraction incorporated at least 5 mol of phosphate per mol of endogenous MAP 2. The kinase phosphorylated MAP 2 with an apparent K_m of 5 μM for ATP and 20 nM for calmodulin. Half-maximal incorporation of phosphate into MAP 2 occurred in <1 min. These results indicate that endogenous MAP 2 is phosphorylated with a high specific activity by the kinase.

We next examined the identity of this endogenous calmodulin-dependent kinase by comparison with the previously purified cytosolic kinase, which contains two autophosphorylating, calmodulin-binding subunits, designated ρ and σ (13). The phosphorylated ρ and σ subunits of the calmodulin-dependent kinase were compared by isoelectric focusing with M_r 52,000–63,000 phosphoproteins in the void fraction of NaCl-treated, chromatographed microtubule protein (Fig. 4 *A* and *B*). There were two phosphoproteins in this molecular weight region that had isoelectric points (pIs) near neutrality and comigrated with the ρ and σ subunits of the purified cytosolic calmodulin-dependent kinase (Fig. 4*B*). In addition, identical phosphopeptide maps were obtained for the cytosolic and void fraction calmodulin-dependent kinases (data not shown). There were also M_r 52,000–55,000 proteins with pIs of 5.4 and 5.3 that comigrated with α and β tubulin, respectively (20, 21). The M_r 280,000 protein had a pI of 5.2 and comigrated

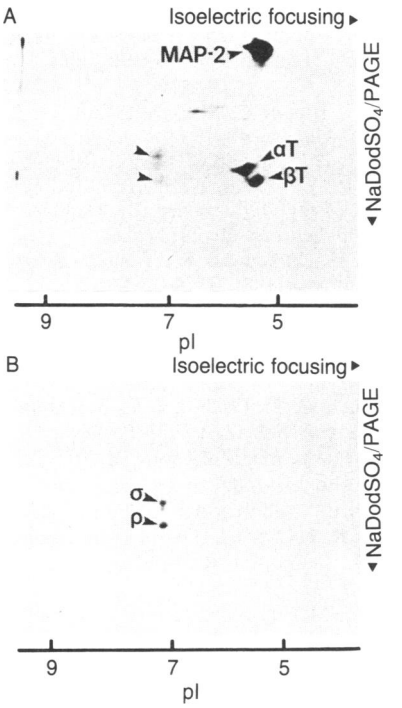

FIG. 4. Identification of major phosphoproteins in microtubules. A void fraction from twice-cycled microtubules was prepared as described in the legend to Fig. 2*B*, phosphorylated in the presence of calcium and calmodulin, and resolved on two-dimensional isoelectric focusing/$NaDodSO_4$/PAGE. (*A*) Autoradiograph of void fraction with the positions of MAP 2, α tubulin (αT), and β tubulin (βT) depicted. (*B*) For comparison, autoradiograph of purified, autophosphorylated ρ and σ enzyme subunits.

with MAP 2. The M_r 80,000–86,000 protein doublet (Fig. 3*B*) did not enter the gel, suggesting that it is either basic or insoluble. We have recently identified this phosphoprotein doublet as synapsin I (unpublished data).

An additional distinctive characteristic of the cytosolic kinase subunits is that they are calmodulin-binding proteins. Twice-cycled microtubule protein contained two calmodulin-binding proteins that comigrated with the ρ and σ subunits of the cytosolic kinase (Fig. 5). When microtubule protein was

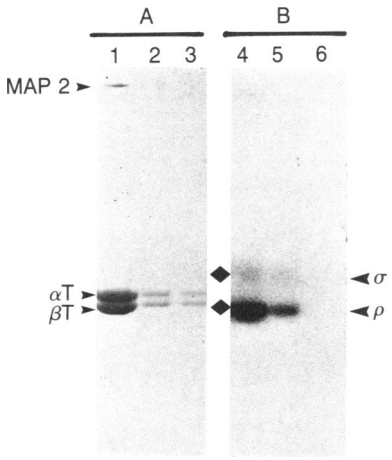

FIG. 5. Calmodulin-binding proteins in microtubules. Protein pattern (*A*, lanes 1–3) and autoradiograph (*B*, lanes 4–6) of twice-cycled microtubule protein (lanes 1 and 4), void (lanes 2 and 5), and back (lanes 3 and 6) fractions prepared as described in the legend to Fig. 2*B*. All samples were assayed for ^{125}I-calmodulin-binding proteins as described (13, 23).

further resolved on Bio-Gel A-15m, the void fraction containing the calmodulin-dependent kinase activity also contained the M_r 52,000–63,000 calmodulin-binding proteins, whereas protein from the back peak was devoid of calmodulin-dependent kinase activity and calmodulin-binding proteins. Two-dimensional gel electrophoresis confirmed that the calmodulin-binding proteins in these fractions have neutral pIs and comigrate with the subunits of the cytosolic calmodulin-dependent kinase (data not shown).

Phosphopeptide Maps of MAP 2 Phosphorylated by cAMP- or Calmodulin-Dependent Kinase. Since both cAMP- and calmodulin-dependent kinases are present in microtubule preparations and utilize MAP 2 as a major substrate, we examined whether these endogenous microtubule-associated kinases phosphorylate unique sites on MAP 2. Two-dimensional thin-layer electrophoresis/chromatography of phosphorylated MAP 2 revealed that distinct phosphopeptides were produced by the two kinases (Fig. 6). The autoradiograph of cAMP-dependent phosphorylation of MAP 2 showed 11 major phosphopeptides (Fig. 6*A*), whereas calmodulin-dependent phosphorylation of MAP 2 produced 5 major phosphopeptides (Fig. 6*B*). For comparison, Fig. 6*C* shows calmodulin-dependent kinase phosphorylation of MAP 2 using calmodulin-dependent kinase purified from cytosol (13). The phosphopeptide maps of MAP 2 phosphorylated by the endogenous microtubule-associated, calmodulin-dependent kinase and the purified cytosolic kinase were identical, indicating that the reconstituted system may be useful in site specificity studies.

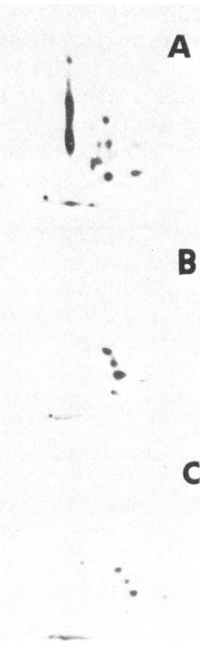

FIG. 6. Phosphopeptide mapping of MAP 2 phosphorylation by cAMP- and calmodulin-dependent kinases endogenous to microtubules. (*A*) MAP 2 phosphorylated by cAMP-dependent kinase in microtubules. (*B*) MAP 2 phosphorylated by calmodulin-dependent kinase in microtubules. Protein from twice-cycled microtubules was incubated in the presence of magnesium and cAMP (*A*) or magnesium, calcium, and calmodulin (*B*) under standard phosphorylating conditions and subsequently resolved on one-dimensional $NaDodSO_4$/PAGE. MAP 2 was excised from the gels and digested with trypsin, and phosphopeptides were resolved on two-dimensional electrophoresis/chromatography. Electrophoresis was from left to right, and chromatography was from the top to the bottom. (*C*) MAP 2 phosphorylated by purified calmodulin-dependent kinase from brain cytosol is shown for comparison with *B*.

DISCUSSION

The data demonstrate that a significant amount of cytosolic calmodulin-dependent kinase is associated with microtubule preparations prepared under conditions that preserve kinase activity. The microtubule-associated calmodulin-dependent kinase is identical to a previously purified cytosolic kinase on the basis of subunit composition, calmodulin-binding proteins, calmodulin-dependent autophosphorylation, apparent K_ms for ATP and calmodulin, substrate specificity, kinetics of phosphorylation, and the pattern of MAP 2 phosphopeptide phosphorylation. An antibody against this kinase specifically labels dendritic microtubules in neurons (25). This enzyme appears to be a type II calcium/calmodulin-dependent protein kinase (14).

MAP 2 is a proposed regulator of the dynamics of microtubule assembly and the interaction of microtubules with other cytoskeletal elements and membrane (1). Phosphorylation of MAP 2 with cAMP-dependent (3) or calmodulin-dependent kinase (4) reduces its microtubule assembly-promoting ability. The demonstration in this report that microtubule preparations contain an endogenous calmodulin-dependent MAP 2 kinase indicates that both calmodulin- and cAMP-dependent kinases may play a role in regulating microtubule function. A previous report indicated that a purified calmodulin-dependent kinase phosphorylated distinct sites on MAP 2 compared to cAMP-dependent kinase (26). In the present report, the endogenous calmodulin-dependent kinase in the void fraction incorporated at least 5 mol of phosphate per mol of endogenous MAP 2. The phosphopeptide maps for cAMP- or calmodulin-dependent phosphorylation of MAP 2 were distinct, suggesting that the two kinases differentially regulate MAP 2 function (Fig. 6*B*). More recent studies utilizing phosphoamino acid analysis in conjunction with comigration analysis suggest that the calmodulin-dependent kinase phosphorylates eight sites on MAP 2 that are distinct from the cAMP-dependent phosphorylation sites (27). Thus, this calmodulin-dependent kinase is a good candidate for the cAMP-independent kinase that phosphorylates MAP 2.

Since the calmodulin-dependent kinase and MAP 2 elute at the void of a Bio-Gel A-15m column, they appear to exist as a high molecular weight structure of >15 million daltons. This population of MAP 2 is separable from the MAP 2 associated with the cAMP-dependent kinase. Future studies are needed to determine the exact constitution of this high molecular weight structure as well as the functional role of the calmodulin-dependent kinase and associated MAP 2 in mediating calcium-regulated changes in microtubule dynamics.

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