A Protein Kinase Bound to the Projection Portion of MAP 2 (Microtubule-associated Protein 2)

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ABSTRACT In previous work we have demonstrated that the microtubule-associated protein 2 (MAP 2) molecule consists of two structural parts. One part of the molecule, referred to as the assembly-promoting domain, binds to the microtubule surface and is responsible for promoting microtubule assembly; the other represents a filamentous projection observed on the microtubule surface that may be involved in the interaction of microtubules with other cellular structures. MAP 2 is known to be specifically phosphorylated as the result of a protein kinase activity that is present in microtubule preparations. We have now found that the activity copurifies with the projection portion of MAP 2 itself. Kinase activity coeluted with MAP 2 when microtubule protein was subjected to either gel-filtration chromatography on Bio-Gel A-15m or ion-exchange chromatography on DEAE-Sephadex. The activity was released from microtubules by mild digestion with chymotrypsin in parallel with the removal by the protease of the MAP 2 projections from the microtubule surface. The association of the activity with the projection was demonstrated directly by gel filtration chromatography of the projections on Bio-Gel A-15m. Three protein species (M_r = 39,000, 55,000, and 70,000) cofractionated with MAP 2, and two of these (M_r = 39,000 and 55,000) may represent the subunits of an associated cyclic AMP-dependent protein kinase. The projection-associated activity was stimulated 10-fold by cyclic AMP and was inhibited >95% by the cyclic AMP-dependent protein kinase inhibitor from rabbit skeletal muscle. It appeared to represent the only significant activity associated with microtubules, almost no activity being found with tubulin, other MAPs, or the assembly-promoting domain of MAP 2, and was estimated to account for 7–22% of the total brain cytosolic protein kinase activity. The location of the kinase on the projection is consistent with a role in regulating the function of the projection, though other roles for the enzyme are also possible.

Microtubules isolated from brain tissue consist of tubulin and a number of microtubule-associated proteins, or MAPs (2, 3, 21, 40, 47). MAP 2 is the most prominent of the MAPs and the most extensively characterized with regard to both structural and functional properties. The MAP 2 molecule has been shown to have two structural domains corresponding to two functional activities that have been ascribed to this protein (43, 44). The larger domain appears as a projection on the microtubule surface and was found to represent ~240,000 daltons of the 270,000-dalton MAP 2 polypeptide chain. The function of this portion of the MAP 2 molecule is not known, but it is likely that it interacts in some way with other components of the cell. These may include membrane-bounded organelles (36), other microtubules or actin filaments (14), or intermediate filaments. Whether the MAP 2 projection domain interacts with other structures in a passive way as part of a cytoskeletal framework, or whether it is actively involved in some motile process in the cell, is a question of considerable interest.

A second, smaller domain of MAP 2, ~35,000 daltons in size, contains the portion of the molecule that binds to the microtubule surface (43). We have referred to this as the assembly-promoting domain because it is responsible for the well-documented in vitro activity of MAP 2 of promoting microtubule assembly (15, 18, 25).

Although work in vitro has suggested two functions for MAP 2, it is not known whether both of these functions are important in the cell. The finding that MAP 2 is subject to phosphorylation in vitro and in vivo (20, 34, 38, 40, 43) has suggested that its activity may be regulated in the cell. The phosphorylation reaction, therefore, is of inherent interest. In addition, investi-

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gation of this reaction may yield some clues as to the function of MAP 2 in vivo. Protein kinase activity has been detected in microtubule and tubulin preparations, and the possible significance of such an activity in controlling microtubule assembly and function has led to numerous attempts at its characterization (8, 13, 16, 28, 34, 37–41). Kinase specific activity was found to become constant during microtubule purification (40), suggesting that at least one form of kinase might be an integral component of microtubules. Further evidence for the association of a protein kinase with microtubules in vivo has come from immunofluorescence microscopy using antibody to the subunits of cyclic AMP(cAMP)–dependent protein kinase (4). The nature of the association of the protein kinase with microtubules and the function of this association is not known.

In an earlier report (43) we showed that both the projection and assembly-promoting domains of MAP 2 contained sites for phosphorylation by the copurifying protein kinase. We noted that removal of the projections from the microtubule surface by mild protease digestion did not inhibit their phosphorylation. This result suggested the interesting possibility that a kinase activity could be associated with the projection itself. The present investigation was initiated to determine whether this is the case. In addition, we hoped to determine whether a projection-associated activity is the sole protein kinase activity present in microtubule preparations. In this report we show that, indeed, almost all of the protein kinase activity in microtubule preparations cofractionates with the MAP 2 projection and we discuss the significance of this finding for the behavior of microtubules in the cell.

MATERIALS AND METHODS

Preparation of Microtubules and MAPs

Microtubule protein was purified from calf cerebral cortex by a modification of the reversible assembly method of Borisy et al. (3). 530 g of brain tissue was homogenized in 800 ml of 0.1 M PIPES, pH 6.6, 0.1 mM EDTA, 1.0 mM EGTA, and 1.0 mM $MgSO_4$ (PEEM buffer) containing 1 mM 2-mercaptoethanol. Homogenization was carried out for 4 s at low speed in a Waring blender (Waring Products Div., Dynamics Corp of America, New Hartford, Conn.) and then at 2,000 rpm in a PotterElvejhem Teflon-pestle homogenizer (Kontes Co., Vineland, N. J.). The homogenate was centrifuged for 90 min at 12,000 rpm in a Sorvall GSA rotor (DuPont Co., Sorvall Biomedical Div., Newtown, Conn.), yielding a brain extract. To purify microtubules, GTP was added to the extract to 1 mM (PEEMG buffer) and the extract was incubated at 37°C for 30 min to allow microtubules to polymerize. Other steps were done as in Borisy et al. (3). One cycle of microtubule purification refers to the assembly and sedimentation of microtubules at 37°C, resuspension in PEEMG and depolymerization of microtubules at 0°C, and recentrifugation at 4°C.

A crude MAP fraction was prepared by chromatography of three-cycle purified microtubule protein on a 1.5 × 2 cm column of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N. J.) that had been preequilibrated with PEEMG buffer containing 0.25 M NaCl. The MAPs eluted unbound from the column.

Fragments of MAP 2

To produce the projection and microtubule assembly–promoting fragments of MAP 2 (43), microtubules at 5 mg/ml in PEEMG buffer were exposed to α-chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.) at 1.0 μg/ml and incubated at 37°C for the periods of time indicated in Results (43). The reaction was stopped with 2 mM phenylmethylsulfonyl fluoride. The microtubules were sedimented at 30,000 *g* at 37°C for 30 min, leaving the MAP 2 projections in the supernate. The assembly-promoting portion of MAP 2 cosedimented with the microtubules along with other MAP species. To prepare the assembly-promoting fragments for chromatography on Bio-Gel A-15m, the sedimented microtubules were resuspended in PEEMG buffer + 0.25 M NaCl to dissociate the fragments from tubulin. The protein was applied to a small column (0.6-ml bed) of DEAE-Sephadex A-50 equilibrated in the same buffer. The assembly-promoting fragments as well as other MAP species eluted unbound from the column while most of the tubulin was retarded. The unbound fraction was applied directly to the A-15 column.

Protein Phosphorylation

The time-course of the endogenous phosphorylation of MAP 2 by the protein kinase activity present in microtubule preparations (see Fig. 1) was determined in a reaction mixture containing 5 mg/ml microtubules in PEEMG buffer, 5 mM $MgSO_4$, 10 μM cAMP, and 25 μM [γ-^{32}P]ATP, which we calculated to represent a five- to sixfold molar excess over MAP 2.

To assay for total protein kinase activity, an exogenous substrate for phosphorylation was added, either calf thymus histone (type IIA; Sigma Chemical Co., St. Louis, Mo.) or heat-inactivated MAP 2. The MAP 2 was prepared by incubating microtubules in a boiling water bath for 5 min (11). Coagulated proteins were removed by centrifugation, leaving MAP 2 (75% of total protein) in the supernate. MAP 2 prepared in this manner is active with regard to its ability to promote microtubule assembly and retains all of its known structural properties (15, 18, 43). In addition, it is phosphorylated at the same sites as untreated MAP 2 (R. Vallee, manuscript in preparation). The one property that we find is destroyed by the exposure to elevated temperatures is its associated protein kinase activity (this paper). Hence, heat-inactivated MAP 2 was deemed suitable for use as a protein kinase substrate in the present investigation. (Heat-treated MAP 2 was also used as a substrate after limited chymotryptic digestion [see Fig. 2].) Protein kinase assays were carried out in PEEM buffer containing 5 mM added $MgSO_4$, 5 μM cAMP (unless otherwise noted), 100 μM [γ-^{32}P]ATP and 1 mg/ml of histone or heat-treated MAP 2. The final volume was 75 μl and the sample volume 10–20 μl. Samples were preincubated at 37°C for 2 min in the presence of $MgSO_4$ and cAMP, and the reaction was initiated by addition of the ATP and the exogenous protein substrate. The time of incubation and the concentration of kinase were adjusted to allow for no more than 1% of the ^{32}P label to be transferred to the substrate.

Incorporation of phosphate into protein was determined in either of two ways: (*a*) using added MAP 2 or added histone as substrate, the reaction was quenched by addition of 2 ml of ice-cold 10% (wt/vol) TCA followed by the addition of 10 μg of bovine serum albumin, and the precipitate obtained was collected by filtration; or (*b*) using endogenous (Fig. 1) or added MAP 2 as substrate, the reaction was quenched by addition of 200 μl of electrophoresis sample buffer (19) to 50 μl of reaction mixture, followed by incubation in a boiling water bath for 1 min. The samples were subjected to electrophoresis (see below) and the radioactive bands excised from stained and dried gels. ^{32}P radioactivity was determined by liquid scintillation counting in Aquasol (New England Nuclear, Boston, Mass.).

cAMP-dependent Protein Kinase and Protein Kinase Inhibitor

The peak I and peak II forms of cAMP-dependent protein kinase from rabbit muscle and the catalytic subunit of cAMP-dependent protein kinase from beef heart, as well as the cAMP-dependent protein kinase inhibitor from rabbit muscle (46), were obtained from Sigma Chemical Co. 1 μg of the inhibitor blocked the cAMP-dependent protein kinase–catalyzed transfer of 0.18 pmol of phosphate/min to casein. The inhibitor preparation was reported to have no detectable phosphodiesterase activating activity and was found by us to have no detectable phosphoprotein phosphatase activity.

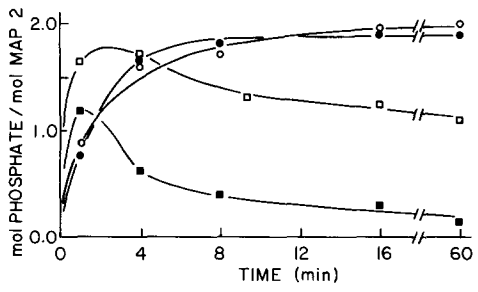

FIGURE 1 Time-course of MAP 2 phosphorylation as a function of the stage of microtubule purification. Microtubules (5 mg/ml) at successive stages of purification were incubated at 37°C in the presence of [γ-^{32}P]ATP, and the incorporation of phosphate into MAP 2 was determined at a series of times. Microtubules were prepared by one (■), two (□), three (●), or four (○) cycles of assembly/disassembly purification.

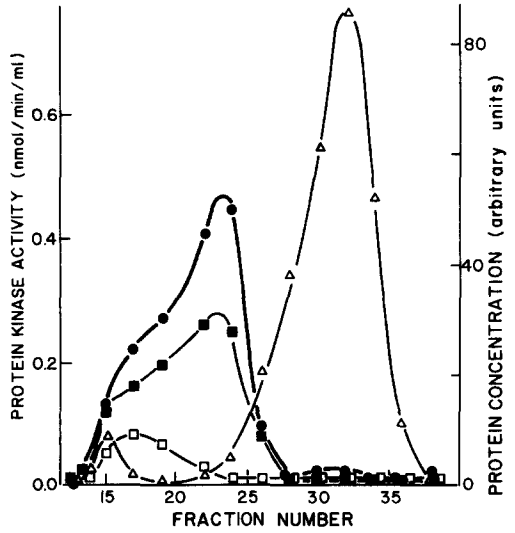

FIGURE 2 Bio-Gel A-15m gel filtration chromatography of microtubule protein. 1.5 ml of microtubule protein (three cycles of purification) at 3.1 mg/ml was applied to a 1.5 × 25 cm column of Bio-Gel A-15m. The column had been preequilibrated with PEEM buffer containing 0.25 M NaCl to dissociate oligomers of tubulin and MAPs (45). The fractions were assayed for protein composition and protein kinase activity. ■, MAP 2; □, MAP 1; △, tubulin; and ●, protein kinase activity. For this experiment alone, partially digested heat-treated MAP 2 was used as the exogenous substrate in the protein kinase assay. Phosphate incorporation into the assembly-promoting and projection fragments of MAP 2 (43) as well as into residual undigested MAP 2 was determined. The distributions of protein kinase activity with all three species as substrate were virtually identical, and, therefore, only the data obtained with intact MAP 2 as substrate are shown. Activity is expressed as nanomoles of phosphate incorporated into MAP 2 per minute per milliliter of column fraction (1-ml fractions). The value obtained for the activity of the MAP 2 peak using total phosphate incorporation into intact MAP 2 + the fragments was 1.8 nmol/min per ml.

Chromatographic Procedures.

Gel filtration chromatography was performed on a 1.5 × 25 cm column of Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, Calif.). Elution volumes were determined by weighing the fractions. Ion-exchange chromatography was performed on a 1 × 5 cm bed of DEAE-Sephadex A-50 preequilibrated with PEEM buffer. Elution of MAP 2 was accomplished using a linear gradient of NaCl. Fractions for both columns were ~1 ml in volume.

Other Analytical Methods

Electrophoresis was conducted in 1.5-mm-thick slab gels, with 9% acrylamide in the running gel (19). Samples were incubated in a boiling water bath for 1 min immediately after mixing with sample buffer. Gels were stained with Coomassie Brilliant Blue R250 (10) or by the silver staining procedure of Merrill et al. (24) as modified by Oakley et al. (27). To determine quantitatively the relative concentrations of the electrophoretic species, samples were loaded in smaller amounts than shown in the figures (<2.5 μg for tubulin, <1 μg for MAP 1 and MAP 2—determined to be the linear range of absorbance as a function of protein concentration) and quantitated by densitometry. Molecular weight was determined using skeletal muscle myosin heavy chain, α-actinin, bovine serum albumin, tubulin, actin, and α-chymotrypsinogen as standards. Protein concentration was determined using bovine serum albumin as standard (22, 35).

RESULTS

Protein Kinase and Phosphoprotein Phosphatase Activity in Microtubule Preparations

As a first step in determining whether protein kinase activity persisted in our microtubule preparations during purification, the time-course of endogenous phosphorylation of MAP 2 was determined for microtubules at consecutive stages in the purification procedure (Fig. 1). With microtubules prepared by one or two purification cycles (1× and 2×), ^{32}P was at first rapidly incorporated into MAP 2 but was subsequently released from the protein. The initial rate of phosphate incorporation decreased with purification but by the third cycle of purification (3×) reached a constant value (compare 3×- and 4×-purified microtubules), consistent with the similar observation of Sloboda et al. (40). Phosphate was now stably incorporated into MAP 2 at a level of ~2 mol of phosphate/mol of MAP 2. (Higher values have been obtained in some preparations.) We interpret these results to indicate the presence of competing phosphoprotein phosphatase and protein kinase activities in the early stages of microtubule purification. Net incorporation of phosphate resulting from the kinase activity would be observed until the supply of ATP was depleted, after which dephosphorylation by the phosphatase would be seen. The phosphoprotein phosphatase activity was apparently lost as microtubules were purified. However, a protein kinase activity was retained and this activity copurified with microtubules.

Cofractionation of Kinase Activity with MAP 2

To determine the molecular nature of the kinase, microtubule protein prepared by three cycles of assembly/disassembly purification was analyzed by gel-filtration chromatography on Bio-Gel A-15m (Figs. 2 and 3). Chromatography was carried out in the presence of 0.25 M NaCl, which disaggregates the tubulin and MAP-containing oligomers (45) present in microtubule preparations. Protein kinase activity was assayed using heat-treated MAP 2 (see Protein Phosphorylation under Materials and Methods) which was added as an exogenous substrate to each fraction. In this particular experiment the MAP 2 substrate was exposed to chymotrypsin for a brief period of time before use to yield a mixture containing a small amount of residual intact protein as well as fragments corresponding to the assembly-promoting and projection domains (43). In this way it could be determined whether separate protein kinase activities responsible for phosphorylating each of the two MAP

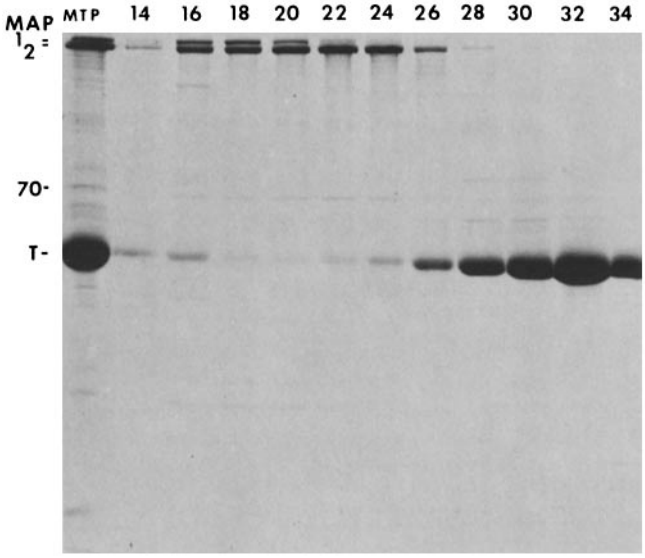

FIGURE 3 Bio-Gel A-15m gel filtration chromatography of microtubule protein. An electrophoretic gel showing column fractions from the experiment described in Fig. 2. Molecular weight values are in thousands. *T* indicates tubulin.

2 domains might be distinguished. The distribution of activity was, in fact, found to be the same with either the individual fragments or the intact protein as substrate. Only the values obtained with intact MAP 2 as substrate are shown (Fig. 2).

Tubulin was found primarily in the low molecular weight region of the column profile (Figs. 2 and 3). Proteins running slightly above tubulin on the SDS gel (55,000–80,000 mol wt) shown in Fig. 3 (fractions 28–32) which may correspond to the MAPs "τ" (47) and "tubulin assembly protein" (21) eluted slightly in advance of tubulin. MAP 2 showed a bimodal distribution with a principal peak eluting midway through the column (K_D = 0.3) and a shoulder near the void volume. The shoulder apparently represents an aggregated form of MAP 2 and is observed in variable amounts in different preparations. MAP 1 eluted as a single skewed peak near the void volume. A pair of proteins of ~30,000 M_r that behave as MAPs and probably represent the "LMW MAPs" identified by Berkowitz et al. (2) eluted near the void volume. These proteins appeared to coelute with MAP 1, though it is not clear whether this reflects the existence of a MAP 1/LMW MAP complex. A band of 70,000 mol wt has consistently been found to cofractionate with MAP 2 and may, in fact, be associated with MAP 2. A species of 39,000 mol wt has also been observed to cofractionate with MAP 2, and this species may represent a MAP 2 bound protein kinase (see below).

The distribution of protein kinase activity (Fig. 2) closely followed the distribution of MAP 2. The position of the peak of activity corresponded to the position of the MAP 2 peak. This elution behavior was in striking contrast to that for free cytosolic cAMP-dependent protein kinase on the same column. The free enzyme eluted as a single peak at about the position of tubulin. This was consistent with a reported Stokes radius for the free enzyme of 6.0 nm (9) in contrast to MAP 2, a highly asymmetric molecule with a Stokes radius that we estimate from sedimentation data (45) to be at least 15 nm (see also reference 5). In addition to the major peak of protein kinase activity, a shoulder in the distribution was observed near the void volume that corresponded to the shoulder in the MAP 2 distribution. It was clear that the distribution of activity did not follow the distribution of MAP 1, nor was much activity found at the position of the proteins identified as τ and tubulin assembly protein (fractions 28–32). A small peak of activity was detected with the tubulin peak. This minor peak may represent free residual cytosolic cAMP-dependent protein kinase. Whether this activity corresponded to an activity previously found in preparations of purified tubulin (13) is not clear. It was evident, however, that most of the protein kinase activity in the preparation (ranging from 90 to 95% in separate experiments) cofractionated with MAP 2.

To test further whether the kinase activity was associated with MAP 2, the peak fractions from an A-15m column profile similar to that shown in Figs. 2 and 3 were pooled and subjected to ion-exchange chromatography on DEAE-Sephadex A-50 (Figs. 4 and 5). MAP 2 eluted as a single peak located at 0.08 M NaCl. A trace amount of MAP 1 eluted as a minor peak centered four fractions after the MAP 2 peak, and trace amounts of tubulin began to elute near the end of the salt gradient. The peak of protein kinase activity coincided with the MAP 2 peak. No other activity was detected in the elution profile. Thus, using separation procedures based on affinity for microtubules (Fig. 1), molecular radius (Figs. 2 and 3), or molecular charge (Figs. 4 and 5), we found that the kinase activity cofractionated with MAP 2.

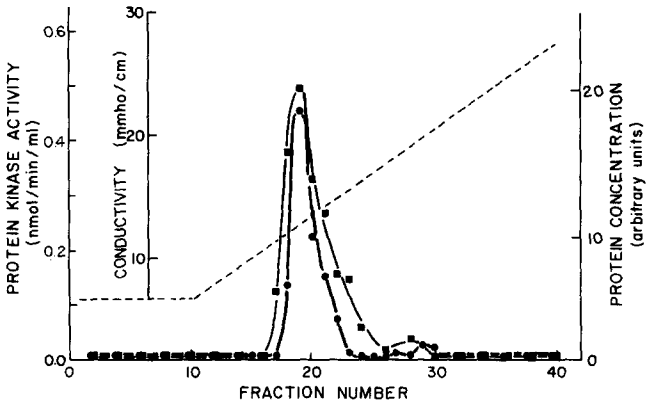

FIGURE 4 Ion exchange chromatography of MAP 2. Four fractions from the major MAP 2 peak (K_D = 0.3) of a Bio-Gel A-15m gel filtration column similar to that shown in Fig. 2 were pooled and dialysed against PEEM buffer. The sample was applied to a 1 × 5 cm column of DEAE-Sephadex A-50 preequilibrated with PEEM buffer and eluted with a 0–0.3 M gradient of NaCl. 1-ml fractions were collected. ATP (10 μM) was included in the gel filtration elution buffer and was added to the ion-exchange fractions to the same concentration to stabilize protein kinase activity. Heat-treated MAPs were used as the substrate for determining protein kinase activity. The MAP 2 peak fraction contained MAP 2 at 0.025 mg/ml and had a specific activity of 19.4 nmol/min per mg. ■, MAP 2; ●, protein kinase activity; dashed lines, conductivity.

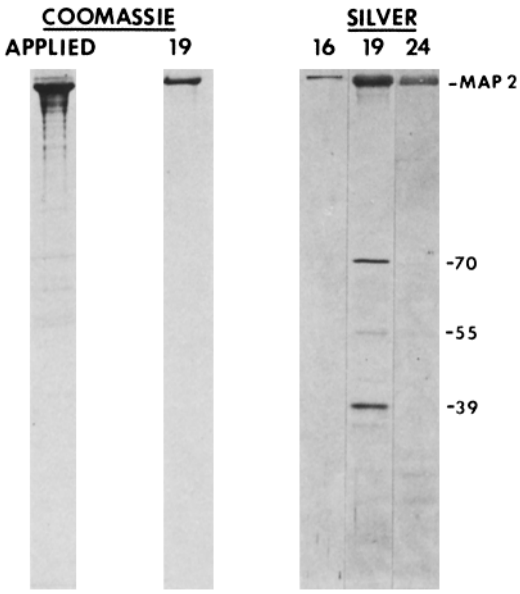

FIGURE 5 Gel electrophoresis of ion-exchange chromatography fractions. The fractions are those described in Fig. 4. The applied sample is shown stained by Coomassie Brilliant Blue (10) as is the peak fraction from the DEAE-Sephadex column (fraction 19, Fig. 4). Fractions from the leading and trailing edges of the column (fractions 16 and 24, respectively) are shown along with the peak fraction, stained by a silver staining procedure (24, 27). Indicated molecular weights are in thousands.

Proteins Associated with MAP 2

In the course of carrying out these experiments, we detected two trace electrophoretic bands of 70,000 and 39,000 M_r that appeared to copurify with MAP 2. These bands were particularly apparent (Fig. 5) after staining gels of purified MAP 2 by use of a highly sensitive silver staining procedure (24, 27). In

addition to the species mentioned, a band at 55,000 mol wt was also detected. The species at 39,000 mol wt coelectrophoresed with the catalytic subunit of cAMP-dependent protein kinase. We believe that this species is, in fact, the catalytic subunit of a MAP 2 bound enzyme. In view of the similarity in its molecular weight to that of the regulatory subunit (R^2) of the peak II form of cAMP-dependent protein kinase (30), the 55,000 mol wt species may represent the regulatory subunit of such an enzyme. Although the 55,000 mol wt species could represent a trace amount of tubulin, it clearly eluted as a peak (cf. lanes *16*, *19*, and *24* of Fig. 5) that was distinct from the principal tubulin peak that began to elute late in the DEAE Sephadex salt gradient (Figs. 4 and 5). The species of 70,000 mol wt has not been identified previously as a component of microtubule preparations. Whether it represents a component of a protein kinase complex or an independent species is not yet apparent. All three species shown in Fig. 5 (fraction 19) were also found in purified preparations of MAP 2 projections (see below).

Thus, we believe that the kinase activity associated with MAP 2 is attributable to a separate molecule, possibly related to the peak II form of cAMP-dependent protein kinase. In support of this possibility was the observation that as much as 80% of the protein kinase activity that cofractionated with MAP 2 was displaced to a position near the salt elution volume of a Bio-Gel A-15m column when cAMP was included in the elution buffer (W. Theurkauf and R. Vallee, unpublished observation). This not only indicates that the kinase activity is attributable to a molecular species distinct from MAP 2, but provides information on the nature of the kinase–MAP 2 association (see Discussion). On the basis of Coomassie Blue staining of the 39,000 mol wt species, which we can detect on heavily loaded gels, and assuming that this species is part of a protein kinase holoenzyme (see reference 26), we estimate that in our purified MAP 2 preparations there are one to two enzyme molecules per every 100 molecules of MAP 2.

Localization of the Kinase Activity on the MAP 2 Molecule

To determine whether the kinase activity was associated with the projection or the assembly-promoting domain of MAP 2 (43), 3×-purified microtubules were exposed to chymotrypsin for a series of times and the microtubules were then sedimented (Fig. 6). Kinase activity was assayed in the supernates, which contained the projection fragments, and in the resuspended microtubule pellets, which contained the assembly-promoting fragments.

Before digestion, 84% of the kinase activity was found in the microtubule pellet, consistent with a strong association of the activity with MAP 2 (Figs. 2–5). As MAP 2 was digested, the kinase activity was released into the supernate. The loss of activity from the microtubule pellets and the increase in activity in the supernates followed a time-course similar to that for the disappearance of the MAP 2 electrophoretic species. By 8 min of digestion, almost all of the activity (90%) was in the supernate. This suggests that most of the kinase activity was associated with the projection portion of the MAP 2 molecule. (It may be noted that total activity was increased by exposure of the microtubules to chymotrypsin. This may reflect an increase in the mobility of the projection upon release from the microtubule surface. Whatever the cause of this phenomenon, it is evident that the increase in the supernate activity is many times

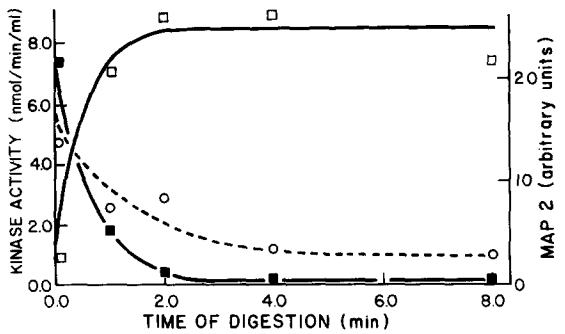

FIGURE 6 Release of protein kinase activity by exposure of microtubules to chymotrypsin. Microtubules (5 mg/ml) were prepared by three cycles of assembly/disassembly purification and then exposed to α-chymotrypsin (1.0 μg/ml) in PEEMG buffer at 37°C. Aliquots were removed at a series of times, and the reaction was quenched with 2 mM phenylmethylsulfonyl fluoride. Samples were taken for electrophoresis at this stage. The microtubules were then sedimented at 30,000 g for 30 min. The supernates contained the MAP 2 projections (43). The pellets were resuspended to the original volume and the microtubules were depolymerized at 0°C. This yielded a pellet fraction that contained the MAP 2 assembly-promoting fragments (43). Protein kinase activity was assayed using an exogenous substrate, histone. (■) MAP 2 total in digest; protein kinase activity (○) in pellet and (□) in supernate.

greater than the increase in total activity and, therefore, does not simply reflect the increase in total activity.)

To determine the location of the kinase activity on the MAP 2 molecule more directly, separate preparations of microtubule-binding and nonbinding fragments of MAP 2 were subjected to gel-filtration chromatography on Bio-Gel A-15m (Figs. 7 and 8). The elution pattern of the microtubule-binding fragments is shown in Figs. 7*A* and 8*A*. The assembly-promoting fragments—microtubule-binding fragments of 32,000 and 34,000 M_r, corresponding to the size expected for the entire assembly-promoting domain of MAP 2 (43)—eluted predominantly as a single peak centered at fraction 31. Protein kinase activity (Fig. 7*A*) showed three peaks, one at the void volume, one at the position of intact MAP 2, and one slightly in advance of the assembly-promoting fragments. The first two peaks appear to represent activity associated with intact MAP 2 (cf. Fig. 2) and possibly with minor high molecular weight microtubule-binding fragments of MAP 2 (43). The third peak did not coincide with any of the species examined. An association of protein kinase activity with the assembly-promoting fragments of MAP 2 was not apparent.

The elution pattern of the MAP 2 fragments that were released from microtubules by exposure to chymotrypsin is shown in Figs. 7*B* and 8*B*. Fragments of 240,000, 180,000, and 140,000 M_r eluted midway through the column. The fragment corresponding to the entire projection portion of MAP 2 (240,000 M_r) was partially resolved on the column from fragments of 180,000 and 140,000 M_r which represent sequentially produced subfragments of the projection (43). The distribution of the 240,000 mol wt fragment as well as the sums of the distributions of the 240,000 and 180,000 mol wt fragments and of all three fragments were determined and are shown in Fig. 7*B* (open, filled, and inverted triangles). A major peak of protein kinase activity was observed in the region of the column profile occupied by the fragments. The peak of activity was shifted from the normal position corresponding to intact MAP 2 (Fig. 2; Fig. 7*A*) to a position corresponding to a species of somewhat smaller size.

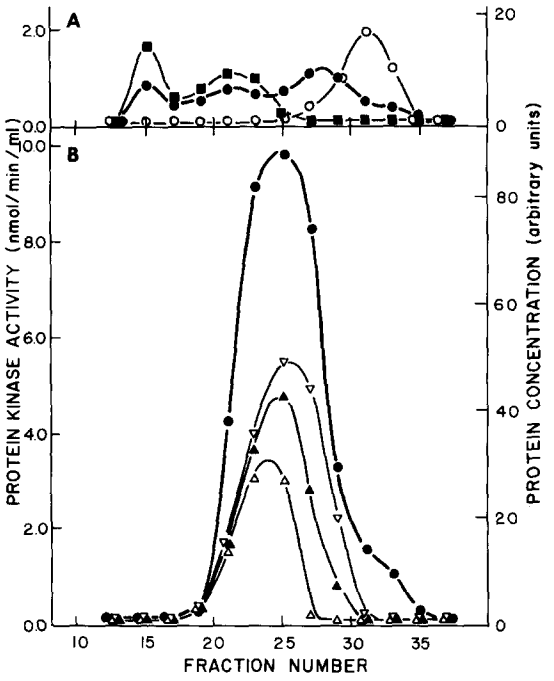

FIGURE 7 Bio-Gel A-15m gel filtration chromatography of MAP 2 fragments. Microtubule protein (3.0 ml at 5 mg/ml) prepared by three cycles of assembly/disassembly purification was exposed to α-chymotrypsin at 1.0 μg/ml for 4 min and the reaction was stopped with 2 mM phenylmethylsulfonyl fluoride. The microtubules were then sedimented at 30,000 g for 30 min. Tubulin was partially removed from the pellet fraction by passage over DEAE-Sephadex A-50 (see Materials and Methods). The pellet fraction contained the MAP 2 assembly-promoting fragments (43), some residual MAP 2, other MAPs, and some tubulin. The supernatant fraction contained the MAP 2 projections, subfragments of the projection, and some tubulin. Bio-Gel A-15m chromatography was performed in PEEM buffer using 1.5 ml of the pellet fraction at 3.9 mg/ml (representing the entire pellet fraction) and 2.0 ml of the supernatant fraction at 1.8 mg/ml (representing two thirds of the total supernatant fraction). Protein kinase activity (●) was assayed using heat-treated MAP 2 as an exogenous substrate added to each column fraction sample. (*A*) Pellet fraction. (○) Assembly-promoting fragments, M_r = 32,000 and 34,000; (■) residual MAP 2. (*B*) Supernatant fraction. (△) Projection fragment, M_r = 240,000; (▲) Projection fragment + subfragment, M_r = 180,000; (▽) projection fragment + subfragments, M_r = 180,000 and 140,000.

To demonstrate an association of the kinase activity with the projection portion of MAP 2 directly, material from the leading edge of an activity peak from a repeat of the experiment depicted in Figs. 7 and 8 was rechromatographed on Bio-Gel A-15m. The pooled fractions were enriched in the 240,000 M_r fragment. This fragment was previously identified as representing in its entirety the portion of MAP 2 that is observed as a projection on the microtubule surface in the electron microscope (43, 44). The results of the rechromatography are shown in Fig. 9. The projection fragment (M_r = 240,000) eluted as a single peak, slightly behind tbe position of intact MAP 2. The peak of protein kinase activity coincided with the projection peak. Hence, we conclude that the protein kinase activity is, indeed, associated with the MAP 2 projection. We note that the peak of activity was sharper than the peak of protein concentration. We believe that this reflects a deviation from linearity that we have observed in the protein kinase assay at low enzyme concentrations (M. DiBartolomeis and R. Vallee, unpublished observation). Constant specific activity across the MAP 2 peak was detected in other experiments not shown here.

Also shown in Fig. 9 are the elution positions of the free peak I and peak II forms of cAMP-dependent protein kinase (*I*, *II*). Both isozymes eluted at the same position in the column (±0.5 ml) separated by many fractions from MAP 2 and the projection fragment. Using the silver staining procedure described earlier (see Fig. 5), the 70,000, 55,000, and 39,000 mol wt species were again detected in the projection peak.

Properties of the MAP 2–Associated Protein Kinase

A variety of results have been reported regarding the degree of dependence on cAMP of protein kinase activity found in microtubule or tubulin preparations (8, 13, 28, 34, 37, 38, 40, 41). The possible presence of cAMP-independent enzymes in these preparations has been raised (33, 37). The MAP 2–associated enzyme that we have identified (Bio-Gel A-15m

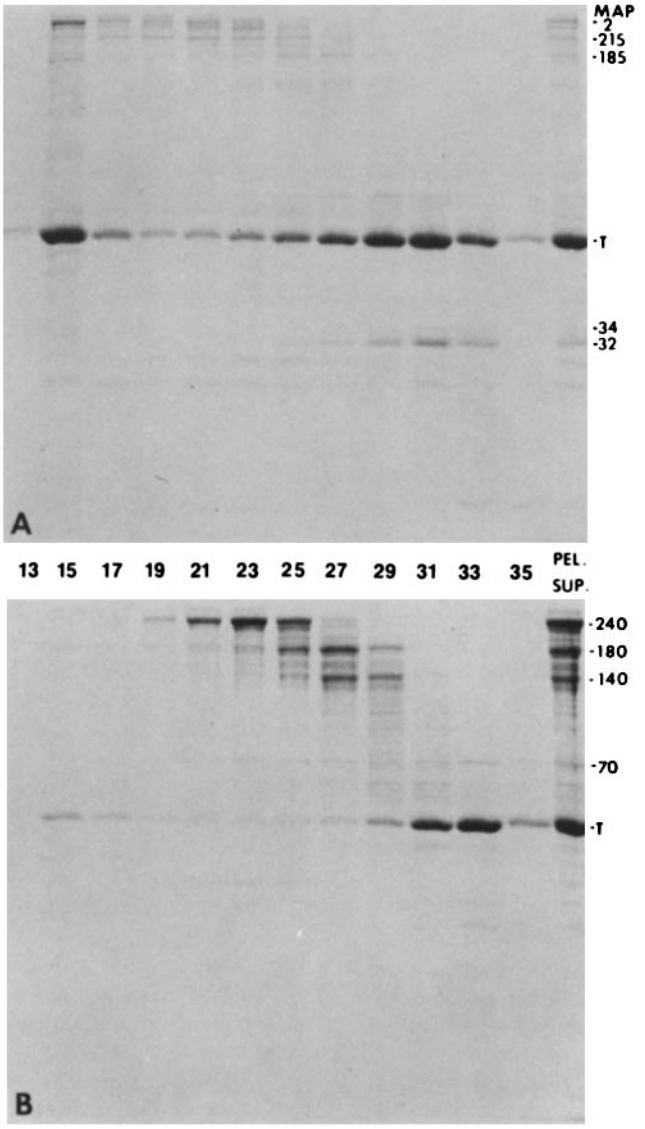

FIGURE 8 Bio-Gel A-15m gel filtration chromatography of MAP 2 fragments. Electrophoretic gels of pellet (*A*) and supernatant (*B*) column fractions, from experiment shown in Fig. 5.

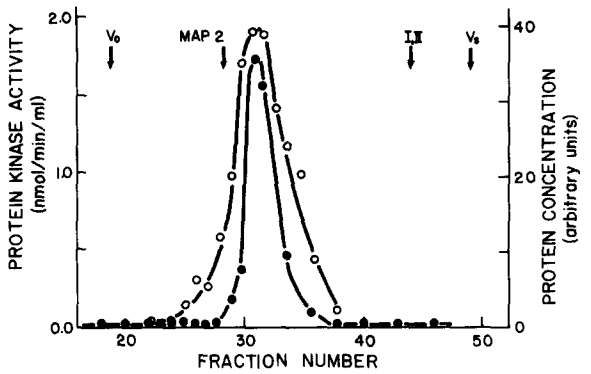

FIGURE 9 Rechromatography of the projection fragment on Bio-Gel A-15m. Fragments of MAP 2 were prepared by digestion of microtubules at 25 mg/ml with 5.0 μg/ml chymotrypsin for 1.5 min. After sedimentation of the microtubules, the supernate (1 ml at 3 mg/ml) containing the projection fragment of MAP 2 and subfragments of the projection was applied to a column of Bio-Gel A-15m. Fractions (2.0 ml) from the leading edge of the protein kinase peak (see Fig. 7) containing primarily the fragment of M_r = 240,000 were pooled and rechromatographed on the same column. PEEM buffer containing 0.25 M NaCl was used for both chromatographic runs. Protein kinase activity was assayed using as substrate heat-treated MAP 2 added to each column fraction sample. 70% of the applied activity was recovered in the rechromatographed projection peak. The concentration of the projection in the peak fraction was 0.05 mg/ml, and the specific activity was 35 nmol/min/mg. (●) Protein kinase activity; (○) the "projection fragment", of M_r = 240,000, representing the entire MAP 2 projection as it is observed by electron microscopy (43).

peak fractions) showed a 10.2-fold increase in activity in the presence of 7 μM cAMP. Thus, the extent of cAMP stimulation was higher in the purified MAP 2 preparation than in whole microtubule protein (40, 43). To confirm the cAMP dependence of the enzyme, the cAMP-dependent protein kinase inhibitor of Walsh et al. (46) was used. Addition of the inhibitor in increasing amounts to a column-purified MAP 2 sample resulted in the almost complete abolition of protein kinase activity (>95% inhibition with 100 μg of the inhibitor fraction added to 4 μg of MAP 2). Thus, the activity associated with MAP 2 is apparently that of a single, cAMP-dependent enzyme.

The specific activity of a column-purified sample of MAP 2 was determined using histone as a substrate. The specific activity was 7.2 nmol/min per mg of the MAP 2/kinase preparation. (It may be noted that the activity of the MAP 2–associated enzyme determined using heat-treated MAPs as substrate (see legends to Figs. 4 and 9) was in general considerably higher than the activity determined using histone.) The brain extract prepared in the first step of microtubule purification and exchanged by Sephadex G-25 chromatography into PEEM buffer + cAMP had an activity of 26 nmol/min per ml when the same substrate was used. From the maximum extent of polymerization that we have observed in the first cycle of microtubule purification, we estimate that there is at least 1 mg/ml of complete microtubule protein in the extract, corresponding to ~0.25 mg/ml of MAP 2. From these values, we obtain a minimum estimate of the total cytosolic protein kinase activity associated with MAP 2 of 1.8 nmol/min per ml, ~7% of the total cytosolic activity. This value may well be an underestimate because of the extensive manipulation of MAP 2 that preceded the measurement of its associated kinase activity. An indication that the activity associated with MAP 2 in the cytosol may be considerably higher came from chromatography of a sample of brain extract on Bio-Gel A-15m. A peak of protein kinase activity was detected at the position expected for MAP 2. This peak accounted for 22% of the cytosolic activity. Thus, it appears that the MAP 2–associated activity represents a significant fraction of the total activity in the brain.

DISCUSSION

Numerous reports have appeared regarding the nature of the protein kinase present in microtubule or tubulin preparations (8, 13, 16, 28, 34, 37–41). We have now found that a significant fraction of the protein kinase activity in brain cytosol and virtually all of the activity in purified microtubules cofractionates with MAP 2. Evidence for the association of the kinase activity with MAP 2 was obtained using a number of fractionation procedures based on totally distinct molecular properties: (*a*) The kinase copurified with microtubules (Fig. 1; Fig. 6, 0-min time-point), indicating that, like MAP 2 (2, 25, 40), it is an integral component of the microtubule polymer. (*b*) The peak of kinase activity coincided with the MAP 2 peak after gel filtration chromatography (Fig. 2), indicating that the activity is associated with a species of the same molecular size as MAP 2. In addition, the bimodal distribution observed for MAP 2 was also observed for the kinase. (*c*) The activity coeluted with MAP 2 by ion-exchange chromatography (Fig. 4), indicating that it is associated with a species that has the same molecular charge as MAP 2. Taken together, these results constitute strong evidence that the kinase activity is associated with MAP 2 itself.

Our earlier work (43) indicated that the primary site of digestion of the MAP 2 molecule by chymotrypsin is at the juncture between the projection and assembly-promoting domains of the molecule. Cleavage by chymotrypsin at this site is closely reflected in the overall rate of disappearance of the MAP 2 electrophoretic species and results in the release of the projection domain from the microtubule surface. In the present study, we observed that almost all of the protein kinase activity associated with microtubules was released from the microtubules with a time-course that closely paralleled the loss of MAP 2 (Fig. 6). This strongly suggested, therefore, that the kinase activity is associated with the projection domain of MAP 2. Direct evidence for this association came from gel-filtration chromatography (Figs. 7–9). It was found that the activity was shifted from the position of intact MAP 2 and coeluted with fragments representing the projection portion of the MAP 2 molecule. Thus, we conclude that the MAP 2 kinase activity is, in fact, associated with the MAP 2 projection.

We obtained no strong evidence for other molecular species in our microtubule preparations having protein kinase activity. A small amount of activity was detected in microtubule pellets after the removal of the MAP 2 projections (Figs. 6 and 7). Much of this activity was ascribed to residual intact MAP 2 and high molecular weight MAP 2 fragments. The remaining activity eluted in advance of the assembly-promoting fragments and did not seem to be specifically associated with them. A small peak of activity was found in association with the tubulin gel-filtration peak (Fig. 2). We do not believe that this represents a tubulin-associated kinase (13), however, because no activity was found associated with tubulin by ion-exchange chromatography (data not shown). The activity that was detected under the tubulin gel-filtration peak may instead repre-

sent contamination with residual cytoplasmic protein kinase activity, which showed a major peak at about the position of tubulin (see Fig. 9).

Molecular Properties of the cAMP-dependent MAP 2 Kinase

The projection-associated activity had the properties of a cAMP-dependent holoenzyme (42). It was dramatically stimulated by cAMP and was almost completely blocked by the inhibitor protein of Walsh et al. (46). There was, therefore, no evidence for a cAMP-independent activity in our preparation of the type recently found associated with neurofilaments (33).

We assume that the protein kinase activity associated with MAP 2 is that of an associated enzyme, probably related to the free cytosolic cAMP-dependent protein kinase (see reference 26). This assumption is based in large part on our observation that as much as 80% of the MAP 2–associated activity was released from MAP 2 as the result of gel-filtration chromatography in the presence of 1.0 μM cAMP (W. Theurkauf and R. Vallee, unpublished results). In addition, we have detected an 39,000 M_r species that cofractionates with MAP 2 and that was indistinguishable by electrophoresis from the catalytic subunit of cAMP-dependent protein kinase (Fig. 5). Together, these results suggest that the MAP 2–associated activity may derive from a bound catalytic subunit similar, at least in size, to that of the cytosolic enzyme. The MAP 2 bound enzyme could be similar to a membrane-bound protein kinase obtained from brain that is structurally homologous, if not identical, to the free peak II cytosolic protein kinase, (7, 31, 32). The catalytic subunit of the membrane-bound enzyme may be released by exposure to cAMP as we have found for the MAP 2 bound enzyme. The membrane-bound species has a regulatory subunit that is closely related to that of the free cytosolic enzyme. Whether this will prove to be the case for the MAP 2 bound enzyme is not yet clear. A 55,000 mol wt species that cofractionated with MAP 2 (Fig. 5) seems a likely candidate for a regulatory subunit in view of its similarity in size to the R^2 regulatory subunit of cAMP-dependent protein kinase (1, 30). The identity of a 70,000 mol wt species that may also be associated with MAP 2 (Fig. 5) is not known. Perhaps this species acts as an adaptor that allows for and limits the association of cAMP-dependent protein kinase with MAP 2.

Despite the evidence that the MAP 2–associated kinase activity resides in an associated enzyme molecule, we have not detected a difference in the elution position of the bulk of MAP 2 and enzymatically active MAP 2. This could reflect a rapid exchange of the associated kinase with MAP 2 resulting in equivalent behavior for all MAP 2 molecules in the preparation. On the other hand, cofractionation could arise simply as the result of the particular physical properties of the proteins in question. The Stokes radius of the highly asymmetric MAP 2 molecule, reflected in the elution position on Bio-Gel A-15m, might be little altered by the binding of a globular protein. The elution behavior on DEAE-Sephadex of a protein kinase bound to MAP 2 could be quite similar to that of MAP 2 if the kinase itself had a weaker affinity for DEAE-Sephadex than did MAP 2 and was, therefore, bound to the column via MAP 2.

The Function of cAMP-dependent MAP 2 Kinase

Phosphorylation may be envisioned to have an effect on either the microtubule assembly–promoting activity of MAP 2 or on the interaction of MAP 2 with other cellular organelles. Two possibly related effects of the cAMP-dependent phosphorylation of MAP 2 on microtubule assembly in vitro have been described (17, 23). (It should be noted that one of these effects, an inhibition of microtubule assembly [17], may be inconsistent with the known effects of cAMP on living cells [12, 29, 48].) Phosphorylation of the projection portion as well as the assembly-promoting portion of MAP 2 has been observed in vitro (43). This suggested that, in addition to playing a role in microtubule assembly, phosphorylation might regulate the interaction of MAP 2 with other cellular components. The present results indicating an association of a protein kinase with the projection portion of MAP 2 would seem to support the latter function for phosphorylation. However, it is still possible, though perhaps less likely, that the projection-bound kinase acts on the assembly-promoting region of MAP 2 in the cell as well. We are presently attempting to determine whether both portions of MAP 2 are phosphorylated in vivo as they are in vitro, to help elucidate the biological role of the phosphorylation of MAP 2.

Whether or not phosphorylation plays a role in regulating microtubule assembly, we are intrigued by the possible implications of our findings for the behavior of the MAP 2 projection in the cell. Rather than representing a passive element of the cytoskeleton, the association of the MAP 2 projection with other cellular components may possibly be reversible. This view is supported by the reversibility of the phosphorylation of MAP 2 at least in vitro (Fig. 1) and by the very existence of the kinase/MAP 2 complex, which presumably would not be required if phosphorylation were irreversible. The reversible association of the MAP 2 projection with other elements of the cytoplasm could be the basis for any of a number of motile phenomena in the cell. Whether the kinase/MAP 2 complex plays a role only in neuronal cells (the major source of MAP 2)—perhaps in axonal or dendritic transport—or in a variety of cell types remains to be determined.

It is equally possible that the localization of the protein kinase on MAP 2 serves some other purpose in the cell, perhaps unrelated to the function of MAP 2 itself. Whatever proves to be the case, it now appears that the MAP 2 molecule has a degree of complexity previously unexpected. Whether this is the case for MAPs in general remains to be determined.

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