Calcium/Phospholipid-dependent Kinase Recognizes Sites in Microtubule-associated Protein 2 Which Are Phosphorylated in Living Brain and Are Not Accessible to Other Kinases*

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Microtubule-associated protein 2 (MAP-2) purified after microtubule assembly cycles from bovine brain had been shown to contain about 10 esterified phosphates (mol/mol), which were relatively phosphate resistant and essentially confined to the projection domain which contributes to the visible arms of microtubules. The kinase responsible for phosphorylating these sites had not been identified. We have approached this question by using a phosphatase that releases the bulk of these residues and then determining which kinase can now add additional residues corresponding to those released. Three kinases were chosen because of their abundance in brain and/or proximity to microtubules. Of these only Ca/phospholipid-dependent kinase was able to recognize the previously occupied sites. We also found that MAP-2 isolated from rat brain without assembly cycles contained more phosphate than previously recognized, >30 mol/mol, suggesting that 20 of these had been inadvertently released by phosphatase during assembly cycles. All 3 kinases (Ca/phospholipid-dependent, cAMP-dependent, and Ca/calmodulin-dependent kinase II) recognized more sites in the bovine than in the rat MAP-2.

When MAP-2, the conspicuous 270-kDa microtubule-associated protein in mammalian brain, is purified after passage through several cycles of temperature-dependent assembly, it contains about 10 mol of phosphate/mol (1–3). When these residues (termed the A subset) were phosphorylated by turnover in living brain, the labeled phosphate proved resistant to a variety of protein phosphatases and was essentially confined to the 240-kDa projection domain of the protein (4).

When cyclic microtubule protein is incubated with ATP an additional 10 residues are added by a co-assembling cAMP-dependent kinase, which is complexed to MAP-2 through its neural specific regulatory subunit (5, 6). This phosphatase-labile (3) "B subset" of residues is concentrated in the short microtubule-binding domain of the protein (4, 7).

In this report we address 2 questions concerning MAP-2 phosphorylation in living brain: what kinase(s) may be responsible for adding the A subset, and does cAMP-dependent kinase phosphorylate the B sites as it does in vitro. A preliminary report has appeared in abstract form (8).

EXPERIMENTAL PROCEDURES

MAP-2 Preparations—Procedures have been described for the preparation of calf brain microtubule protein by 2 cycles of temperature-dependent assembly (3) and for the further purification of MAP-2 by a heat step followed by elution from Bio-Gel A-1.5m (4). MAP-2 was phosphorylated in the B subset by incubating twice-cycled microtubule protein with cAMP and (γ-32P)ATP until phosphorylation by the endogenous kinase was maximal, usually 30 min (4). Under our conditions there was no exchange between ATP and the unlabeled A subset phosphates already present in MAP-2 (3). MAP-2 was then purified as above; a typical yield was 7 mg from 100 mg of twice cycled starting material.

Rat brain MAP-2 was rapidly purified, without the warm incubations associated with assembly cycles, as follows. Fifty male 175–200-g Sprague-Dawley rats were decapitated, the brains were removed and placed on ice, and at once (1 min after death) individually homogenized with 4 strokes of a motor-driven serrated Teflon pestle in glass mortar. The buffer (1.5 ml/brain) contained 100 mM K+ MES, pH 6.9, 1 mM EGTA; 0.5 mM MgSO4; 50 mM NaF; 10 mM NaPPi; 2 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation for 60 min at 100,000 × g at 4 °C, the supernatant was supplemented with NaCl to 0.75 M and then diluted 1:1 with identical buffer. The protocol for the heat step was 10 ml of diluted supernatant in a 50-ml conical glass tube/1 liter of actively boiling water bath, 5 min with occasional stirring with a glass rod. The rapidly chilled solution was centrifuged for 45 min at 40,000 × g at +2 °C, and the supernatant (120 ml) was concentrated by adding an equal volume of saturated ammonium sulfate in reassembly buffer (100 mM K+ MES, pH 6.9, 1 mM EGTA, 0.5 mM MgSO4.) The pellet after centrifugation was dissolved in 1.5 ml of reassembly buffer, and incubated for 10 min at 30 °C after adding MgSO4 to make 4 mM, and 15 μg each of RNase A and DNase I. The solution was then applied to a Bio-Gel A-1.5m column (93 × 1.6 cm) and eluted with reassembly buffer containing 2 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 750 mM NaCl. Protein (0.9 mg) was precipitated from the three 3-ml fractions of the first peak, using ammonium sulfate as above. Assay for total phosphate showed 50 mol/mmol of MAP-2. The value was 30 in another preparation where nuclease treatment was omitted, and the interval between death and homogenization was about 10 min, but we do not know whether the variation was related to these changes. The MAP-2 was prepared for use as a kinase substrate by desalting with 20 bed volumes of Sephadex G-50m equilibrated with reassembly buffer, and again concentrating with a Centricon 30 microconcentrator (Amicon Corp. 4209).

Phospholabeling MAP-2 by Turnover in Living Brain—Each lateral ventricle of 15 adult rats (male Sprague-Dawley, 200 g) was injected with 1.5 mCi of 32P; in 1.5 μl of Dulbecco's phosphate-buffered saline by the following procedure. Rats were anesthetized with Penthrane (methoxyflurane), and the head was restrained in a stereotaxic frame.
After washing the head with 70% ethanol, an excision was made through the skin to expose the skull. Two holes were made in the skull, 2 mm to the right and left of the center point of the skull and 1.5 mm caudally, using a 22-gauge needle. The holes were made just deep enough to allow penetration to the brain. Radioactive solution was injected with a 10-μl Hamilton syringe, modified with a stop plate so the needle could not penetrate beyond 4 mm. Sectioning of frozen brain 4 h after similar injection of trypan blue confirmed the intraventricular site. Any bleeding was cauterized with a flame-heated needle. Recovery from anesthesia took about 30 min. The animals were killed by decapitation after 4 h.

Brains were rapidly removed onto ice and homogenized, after about 30 min, as described above into 20 ml of cold reassembly buffer containing 1 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 unit/ml aprotinin. The supernatant obtained after centrifuging for 1 h at 100,000 × g at +2°C was supplemented with carrier unlabeled MAP-2 in the form of 60 mg (3 ml) of twice-cycled bovine microtubule protein. MAP-2 was promptly purified by the procedure described above for rapid isolation from rat brain. Pooled Bio-Gel A-1.5 m eluates (16 ml) were concentrated by adding an equal volume of saturated ammonium sulfate and desalted into reassembly buffer (as described in the preceding section) because of their different distributions between the microtubule-binding and projection domains of MAP-2 (4). The results shown in Fig. 1 confirm that different phosphopeptide fragments are observed after partial protease digestion.

Phosphatase Assays—Phosphorylated substrates were prepared as described above for MAP-2 and previously for histone (3). The amount of 32P released was determined by a solvent extraction procedure (3). Incubation conditions were as described for brain phosphatase I (3) and calcineurin (4). For bulk removal of phosphate by PAGE using gradients of 4-16% acrylamide and 1-8 M urea. The MAP-2 doublet was conspicuously labeled relative to other proteins (MAP-la and -1b are also comparably labeled, while -IC (13) appears not to be). Fig. 3 shows a densitometric scan of lane IP in Fig. 2: 6.7% of the total 32P present was in MAP-2, 8.7% in MAP-1.

Intraventricular injection was adopted, after preliminary trials using brain slices, intracranial injection in newborn rats, or primary culture of fetal rat brain gave disappointing results. Microcurie levels of 32P, had induced very little labeling (Footnote 4 in Ref. 4). That this was not due to selective failure of the A subset to turn over is indicated by PAGE analysis of the crude brain supernatant (Fig. 2). The MAP-2 doublet was conspicuously labeled relative to other proteins (MAP-1a and -1b are also comparably labeled, while -1c (13) appears not to be). Fig. 3 shows a densitometric scan of lane IP in Fig. 2: 6.7% of the total 32P present was in MAP-2, 8.7% in MAP-1.

Results

Phosphorylation of MAP-2 in Living Brain—The phosphates present in MAP-2 purified after preliminary microtubule assembly cycles had been found resistant to several protein phosphatases (4). The first step in searching for a phosphatase that could release this “A subset” of phosphates from the projection domain of the protein was to seek a more effective way of labeling them. Intracerebral injections of rats with 32P, had induced very little labeling (Footnote 4 in Ref. 4). That this was not due to selective failure of the A subset to turn over is indicated by PAGE analysis of the crude brain supernatant (Fig. 2). The MAP-2 doublet was conspicuously labeled relative to other proteins (MAP-1a and -1b are also comparably labeled, while -1c (13) appears not to be). Fig. 3 shows a densitometric scan of lane IP in Fig. 2: 6.7% of the total 32P present was in MAP-2, 8.7% in MAP-1.

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**C Kinase Sites in MAP-2**

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K.-P. Huang, manuscript in preparation.
C Kinase Sites in MAP-2

MAP-1a  MAP-1b  MAP-1c  MAP-2

1S  1P  2S  2P

Fig. 2. PAGE analysis of rat brain supernatant fraction, after intraventricular injection of $^{32}$P. Small (lanes 1) and large (lanes 2) aliquots were applied; S, Coomassie stain; P, autoradiogram.

Fig. 3. Scan of lane 1P in Fig. 1.

TABLE I

| Hours after injection | Per cent of injected $^{32}$P in Acid soluble Phospho-protein Phospholipid |
|-----------------------|---------------------------------|-------------------------------|
| 4                     | 20                              | 0.11                          | 5.1                           |
| 24                    | 11                              | 0.12                          | 6.7                           |

Dephosphorylation of MAP-2—With previous preparations of MAP-2 phosphorylated in vivo, essentially no phosphate was released by 4 different protein phosphatases, under conditions where up to 80% was released from the B subset labeled in vitro (4). Currently the distinction is less black and white; up to one-fourth of the A subset label has been released by brain phosphatase I or calcineurin and two-thirds by a second brain phosphatase (14). However, the most useful enzyme proved to be an intestinal alkaline phosphatase (Boehringer 713-023) preparation, which differed from others of its kind in having no measurable protease activity toward MAP-2. Fig. 4 shows no fragments released after 1 h of incubation with a very high phosphatase concentration, resulting in release of the bulk of the $^{32}$P (in this case from the B subset). Identical results were obtained in the presence or absence of protease inhibitors (Fig. 4), confirmed by densitometric scans of staining in the MAP-2 area (Table II).

This enzyme was also effective in releasing the bulk of the A subset phosphate, about one-third remaining resistant even to large amounts of enzyme or to additional enzyme added in the course of the incubation (Fig. 5). An important question is whether the A subset turns over relatively uniformly after intraventricular injection or whether only a portion becomes radioactive. Consistent with uniform turnover, the time course of release of total phosphate paralleled that of $^{32}$P (Fig. 5).
Absence of protease activity in alkaline phosphatase

MAP-2 (1.1 mg/ml) phospholabeled in B subset was incubated for 60 min at 37 °C with 60 µg/ml alkaline phosphatase and protease inhibitors as indicated. The amount of MAP-2 remaining after the incubation was determined by PAGE analysis and scanning the appropriate peaks after staining.

<table>
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<tr>
<th>Protease inhibitor</th>
<th>32P released</th>
<th>MAP-2 peak area</th>
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<td>Leupeptin</td>
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<td>%</td>
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<td>53</td>
</tr>
<tr>
<td>10</td>
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<td>1</td>
<td>10</td>
<td>59</td>
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Release of B subset phosphates also appeared multiphasic (Fig. 6), and the last third was completely resistant. However, if MAP-2 was first unfolded by heating in thiol and dilute SDS, more than 90% could be released (Fig. 6).

Activity for MAP-2 dephosphorylation was optimal at neutral pH and was not affected by added Zn²⁺.

What Kinase(s) Might Be Responsible for Adding the 10 Phosphates Present in MAP-2 as Isolated after Assembly Cycle—Our initial approach was to remove the bulk of these phosphates with alkaline phosphatase and then determine whether a kinase could add additional residues over and above those it could add to the control MAP-2. cAMP-dependent kinase was an obvious candidate (5), and Ca/calmodulin-dependent protein kinase II and Ca/phospholipid-dependent protein kinase were chosen because they are abundant in brain relative to other tissues.

Fig. 7 shows the time course of phospholabeling of MAP-2, before and after phosphatase treatment, by each of these kinases. Clearly only Ca/phospholipid-dependent protein ki-
**TABLE II**

**Extent to which various kinases can phosphorylate bovine MAP-2, purified after assembly cycles, before and after its phosphate content has been reduced by incubation with alkaline phosphatase.**

Phosphatase was added to MAP-2 and then inactivated by heating either at zero time or after 30 min of incubation, as described under “Experimental Procedures.” Aliquots were incubated with kinase and [32P]ATP. The table shows the mol of 32P introduced after reaching a plateau (less than 5% of labeling could be attributed to autophosphorylation of kinase in each case). Labeling by exchange with ATP was ruled out by redetermining total phosphate after incubations (last column). cAMP K, cAMP-dependent protein kinase; CAM K, Ca/calmodulin-dependent protein kinase II; C K, Ca/phospholipid-dependent protein kinase.

<table>
<thead>
<tr>
<th>Kinase treatment</th>
<th>Time for extent of phosphorylation to reach plateau</th>
<th>Before incubation with kinase</th>
<th>Maximum labeled phosphate added by kinase</th>
<th>Sum</th>
<th>Measured total phosphate after incubation</th>
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<tr>
<td></td>
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<td>-5.7</td>
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**Fig. 8. Time course of MAP-2 phosphorylation by a partially purified peak I of Ca/calmodulin-dependent kinase (1.2 mg/ml).** Phosphatase-treated (Δ, 2.5 mol/mol) and untreated (▲, 8.5 mol/mol) MAP-2; ▼, autophosphorylation of enzyme fraction in the absence of MAP-2 (values arbitrarily expressed as mol/mol MAP-2 to facilitate comparison).

**Fig. 9. Time course of MAP-2 phosphorylation by Ca/phospholipid-dependent protein kinase (30 µg/ml) in the presence (○) and absence (●) of Ca2+ and phosphatidylserine.**

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Phosphorylation could recognize the sites that are phosphorylated in the A subset. The time course of the incremental phosphorylation (panel C, dashed line) was roughly parallel to that of the phosphorylation of other sites. Table III documents that, with 2 different batches of MAP-2, Ca/phospholipid-dependent kinase added extra phosphate corresponding closely to the increment released by phosphatase. The last column shows that no exchange took place between ATP and protein phosphate, since total phosphate equals the sum of 32P plus original phosphate.

We also tested a fraction of Ca/calmodulin-dependent kinase, possibly kinase I (15), that eluted earlier than Ca/calmodulin-dependent kinase II from Sephacryl S-400 (9). It also did not appear to recognize A subset sites (Fig. 8). This partially purified enzyme contained phosphatase and fixed substantial phosphate in the absence of MAP-2 (Fig. 8). Less than 5% of phospholabeling was due to autophosphorylation with the above 3 purified kinases. Phosphorylation of MAP-2 by Ca/phospholipid-dependent kinase was stimulated by Ca2+ and phosphotidylserine (Fig. 9). Complete dependence is also not seen with other substrates at these high (15-75 µg/ml) enzyme concentrations, because initial rates cannot be determined. However, when Ca/phospholipid-dependent kinase was assayed with histone IIIS as substrate, at enzyme concentrations below 0.025 µg/ml, complete dependence on both Ca2+ and phosphatidylserine was observed.

**Sites Accessible to cAMP-dependent Protein Kinase in Vitro May Also Be Phosphorylated in Vivo**—The B subset sites accessible to cAMP kinase in vitro are unoccupied in MAP-2 purified after assembly cycles. This could be because they are never phosphorylated in vivo, or are regulated empty, or are vacated by phosphatase during MAP-2 isolation. To avoid the warm incubation associated with assembly cycles, we purified MAP-2 rapidly from rat brain, as described under "Experi-
mental Procedures." The staining pattern after PAGE analysis is shown in Fig. 10; integration of peak areas indicated 91% purity. This MAP-2 was indeed found to contain much more phosphate than previously observed, 30 and 50 mol/mol in 2 different preparations.

Fig. 11 shows that the batch with 30 mol/mol had a diminished capacity to be phosphorylated, compared to bovine MAP-2 having 8.5 mol/mol. Half of the 10 sites accessible to cAMP-dependent kinase in MAP-2 from assembly cycles appeared to be occupied when it was rapidly isolated in the cold. Two-thirds of the sites appeared to be occupied in the MAP-2 with 50 mol of phosphate/mol (Table IV).

Samples of bovine and rat (30 mol/mol) MAP-2 phosphorylated by cAMP-dependent kinase on the same day were analyzed by PAGE in adjacent wells. From scans, the ratio of autoradiogram area to stain area was determined for each MAP-2 band. The result confirmed a 40% (43% in Table IV) decrease in available sites in the rat sample.

Sites available to the other 2 kinases, particularly Ca/calmodulin-dependent kinase II, also appeared to be reduced in MAP-2 isolated without prior assembly cycles (Fig. 11 and Table IV).

DISCUSSION

Several reports have described rapid phosphorylation of MAP-2 at multiple sites, catalyzed by Ca/calmodulin-dependent kinase II as well as by cAMP-dependent protein kinase (16-20). On the basis of additivity of phosphorylation (17) or 32P distribution among protease fragments (18-20), the kinases appeared to recognize different sites, at least in part, although interpretation was complicated by the large number of sites and large size of the protein. Until now MAP-2 had not been characterized as a Ca/phospholipid-dependent protein kinase substrate, except for a brief mention that addition of Ca²⁺ and phosphatidylserine to crude brain cytosol enhanced its phosphorylation (18).

The question whether the availability of sites might be affected by the heat step used to purify MAP-2 can be answered at least for cAMP-dependent protein kinase. About 10 mol are added in this case either to heated substrate as reported here and previously (21) or to unheated substrate, i.e. by autophosphorylation of cycled microtubule protein (1, 3, 7).

Information about MAP-2 phosphorylation in living brain was confined to the observation that it contained about 10 mol/mol, when purified after assembly cycles (1, 3). This "A subset" could be distinguished from the "B subset" phosphates added in vitro by cAMP-dependent protein kinase in that they were phosphatase resistant and confined to the projection domain (4). There had been no clue as to which kinase had added the A subset.

With a phosphatase free of protease activity toward MAP-2, we have removed the bulk of the A subset and examined the ability of several kinases to add incremental residues proportional to the number removed. cAMP-dependent protein kinase, an obvious candidate because of its association with MAP-2 in brain, was found unable to recognize A subset sites, in confirmation of Theurkauf and Vallee (1). The other 2 kinases were chosen because of their marked abundance in brain. Ca/calmodulin-dependent kinase II, which in fact is even co-localized with MAP-2 in dendritic processes, was also found totally unable to recognize these sites. Ca/phospholipid-
dependent protein kinase, however, consistently added increased membrane phosphate proportional to the amount released by phosphatase. It also recognized many other sites, with roughly the same facility as those of the A subset.

Unexpectedly, we found that MAP-2 rapidly isolated from rat brain contained not 10 (as in bovine MAP-2 purified after assembly cycles) but more than 30 mol of phosphate/mol. MAP-2 phosphorylated in vitro in earlier work (4) had also been purified relatively rapidly, but from rat brains subject to radiation necrosis and without phosphatase inhibitors in the homogenization buffer. It is, therefore, uncertain whether the observed partition of label, 95% in projection and 5% in binding domain, applies to 10 phosphates as we had assumed (4) or to a larger number.

The availability of MAP-2 with either 10 (bovine) or 30 (rat) mol of phosphate provided an opportunity to determine whether the latter had diminished sites accessible to the various kinases. CAMP-dependent protein kinase consistently adds 10 mol to the bovine, about 6 in the binding domain, and 4 in the projection domain (4). We found that only about half as much phosphate could be added to the rat MAP-2 (Fig. 11 and Table IV). Coupled to the intimate association between this kinase and MAP-2 in brain, this could be taken to provide presumptive evidence that CAMP-dependent kinase phosphorylates MAP-2 in living brain, as it does in vitro. Diminution in sites accessible to Ca/phospholipid-dependent kinase was more equivocal. However, the diminution in sites for Ca/calmodulin-dependent kinase was also substantial. Although Ca/calmodulin-dependent kinase is not known to exist as a complex with MAP-2, it is closely co-localized with this substrate in dendritic processes. Thus, either of these kinases might tentatively be considered a candidate to have introduced the additional phosphates present in MAP-2 which has not passed through in vitro assembly cycles.

In conclusion, Ca/phospholipid-dependent kinase is a strong candidate to be the enzyme that introduces the phosphate-resistant A subset of 10 phosphates in the projection domain, and there is presumptive evidence that MAP-2 is also phosphorylated by CAMP-dependent kinase in vivo. Finally, MAP-2 appears to be present in rat brain in a more highly phosphorylated form than had hitherto been realized.

The next step in characterizing MAP-2 phosphorylation in brain might be to incubate slices with second messengers or other specific kinase activators and then rapidly isolate MAP-2 and determine any loss or gain in sites accessible to various kinases (22).

Acknowledgments—We are indebted to Dr. Randy Kincaid for calmodulin, Dr. Marita King for calcineurin, and Dr. Wayne Albers for the stereotaxic frame used for intraventricular injection.

REFERENCES


### TABLE IV

<table>
<thead>
<tr>
<th>Kinase</th>
<th>MAP-2 Substrate</th>
<th>Time for Extent of Phosphorylation to Reach Plateau</th>
<th>Phosphate Content of MAP-2 Before Incubation with Kinase</th>
<th>Maximum Labeled Phosphate Added by Kinase</th>
<th>Decreases of Available Sites in Rat MAP-2</th>
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<td>C K, 30</td>
<td>Bovine</td>
<td>30 min</td>
<td>8.5</td>
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<td>Rat</td>
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