Microtubule-associated protein 2 (MAP 2) is the major substrate for phosphorylation in purified preparations of brain microtubules. In earlier work, we showed that phosphorylation is catalyzed by a type II cAMP-dependent protein kinase tightly associated with MAP 2 itself. In the present study, we have examined the extent of MAP 2 phosphorylation by its associated protein kinase. Using an inorganic phosphate assay, we found that MAP 2 contained from 8 to 13 mol of phosphate/mol of protein as isolated. The catalytic subunit of the MAP 2-associated kinase catalyzed the incorporation of additional phosphate to a final level of 20–22 mol/mol of MAP 2. Potato acid phosphatase was used to remove phosphate from MAP 2. Rephosphorylation of acid phosphatase-treated MAP 2 resulted in maximal incorporation of 13 mol of phosphate/mol of MAP 2. The rates and extent of [32P]phosphate incorporation into isolated and dephosphorylated MAP 2 were found to be identical, and phosphatase was incorporated into identical peptides in the two preparations. These results were interpreted to indicate that MAP 2 contains as many as 13 CAMP-dependent phosphorylation sites, and approximately eight phosphates of as yet undetermined origin.

MAP 2 is the major substrate for endogenous phosphorylation in microtubules purified from brain tissue (Sloboda et al., 1975; Vallee, 1980). We have recently found that this phosphorylation reaction is catalyzed by a type II CAMP-dependent protein kinase that is associated with MAP 2 itself (Vallee et al., 1981; Theurkauf and Vallee, 1982). This enzyme accounted for virtually all of the kinase activity in purified microtubule preparations and represented one third of the total cytosolic CAMP-dependent protein kinase in cytosolic extracts of brain tissue. The enzymatic properties of the MAP 2-associated enzyme were identical with those of other type II kinases and, in particular, the catalytic subunit of the enzyme was indistinguishable from other catalytic subunits.

Widely divergent values for the number of moles of [32P]phosphate incorporated into MAP 2 in vitro have been obtained, ranging from 2 mol/mol (Sloboda et al., 1975; Vallee et al., 1981) to 4 mol/mol (Coughlin et al., 1980) and as high as 7 mol/mol (Islam and Burns, 1981) with even higher values suggested in the latter study. In addition, the degree of dependence of phosphorylation rate and extent on the presence of cAMP has varied considerably from study to study, suggesting the existence of both cAMP-dependent and cAMP-independent enzymes in microtubule preparations.

The present study was undertaken to clarify the nature and identity of phosphorylation sites on MAP 2. To this end we have determined the extent of phosphorylation of MAP 2 as it was isolated and the full extent to which it could be phosphorylated by its associated cAMP-dependent protein kinase. To determine the number of cAMP-dependent phosphorylation sites, we developed a procedure to dephosphorylate MAP 2. To evaluate the full extent of CAMP-dependent phosphorylation, we exposed the dephosphorylated protein to the purified catalytic subunit of the MAP 2-associated CAMP-dependent protein kinase. We find that MAP 2 is extensively phosphorylated as isolated, and can be further phosphorylated at numerous sites by the CAMP-dependent protein kinase alone. However, a significant number of phosphates cannot be readily accounted for by this enzyme, suggesting the possible existence of other MAP 2 kinases.

**MATERIALS AND METHODS**

**Chemicals—**ATP, GTP, cAMP, acid phosphatase from potato (type III), and yeast hexokinase were obtained from Sigma; *Staphylococcus V*-8 protease was obtained from Miles Laboratories, Inc. (Elkhart, IN). Leupeptin and pepstatin A were obtained from Transformation Research (Framingham, MA). α₂-Macroglobulin was purchased from Boehringer Mannheim. Adenosine [γ-32P]triphosphate (10–40 Ci/mmol) was obtained from New England Nuclear. Taxol was a gift of the National Cancer Institute.

**Microtubules and MAPs—**Microtubule protein was prepared from a cytosolic extract of calf cerebral cortex by cycles of assembly/disassembly purification using a polymerization buffer of 0.1 M PIPES, pH 6.6, 1.0 mM EGTA, and 1.0 mM MgSO₄ (PEM buffer) by a modification of the procedure of Borisy et al. (1975) as previously described (Vallee et al., 1981). Microtubules were also prepared by this procedure with the addition of hexokinase at 40 units/ml plus 100 mM glucose to the cytosolic extract just following the first microtubule polymerization step and prior to the first microtubule sedimentation step to deplete the extract of ATP. Alternatively, microtubules were prepared by a one-step procedure using taxol to induce microtubule polymerization (Vallee, 1983), but in the absence of added GTP. Heat-stable MAPs, containing MAP 2 and r (Weingarten et al., 1975), were prepared by the method of Kim et al. (1979). MAP 2 was purified from heat-stable MAPs by chromatography on Bio-Gel A-15m (Vallee, 1980).

**Phosphate Analysis—**100–300 μg of protein in PEM buffer were brought to 50% saturation with ammonium sulfate, incubated for 5 min at 0 °C, and centrifuged for 5 min at 15,000 rpm in an Eppendorf microcentrifuge. Precipitated protein was resuspended with 100 μl of H₂O and aliquots were removed for protein determination. This first step removes most of the nucleotide and PIPES. The resuspended protein was then transferred to heavy walled glass test tubes (10 × 44 mm) and brought to 10% perchloric acid. Precipitated protein was

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TABLE I

Phosphate content of MAP 2

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Sample As isolated</th>
<th>Phosphorylated with C</th>
<th>Increase</th>
<th>Dephosphorylated</th>
<th>Dephosphorylated with C</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-stable MAPs&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A</td>
<td>8.4</td>
<td>21.4</td>
<td>13.0</td>
<td>2.1</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.6</td>
<td>20.0</td>
<td>7.4</td>
<td>3.3</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>13.0</td>
<td>19.5</td>
<td>6.5</td>
<td>1.4</td>
<td>14.7</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>11.3 ± 2.5</td>
<td>20.3 ± 1.0</td>
<td>9.0 ± 3.5</td>
<td>2.3 ± 1.0</td>
<td>12.4 ± 2.1</td>
</tr>
<tr>
<td>Heat-stable MAPs, hexokinase</td>
<td>D</td>
<td>7.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>used during purification&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>6.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-stable MAPs, no GTP used during purification&lt;sup&gt;c&lt;/sup&gt;</td>
<td>E</td>
<td>9.8</td>
<td>21.7</td>
<td>11.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrophotochemically purified MAP 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>F</td>
<td>13.0</td>
<td>9.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.1</td>
<td>9.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Bio-Gel A-15m-purified MAP 2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>G</td>
<td>7.4</td>
<td>11.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.1</td>
<td>11.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>10.5</td>
<td>10.5 ± 0.6</td>
<td>2.6 ± 0.5</td>
<td>10.4 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>10.3 ± 2.8</td>
<td>20.7 ± 1.1</td>
<td>10.0 ± 2.6</td>
<td>2.4 ± 0.8</td>
<td>12.4 ± 2.1</td>
</tr>
<tr>
<td>Average of all methods</td>
<td></td>
<td>9.9 ± 2.5</td>
<td>20.7 ± 1.1</td>
<td>10.0 ± 2.6</td>
<td>2.4 ± 0.8</td>
<td>12.4 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values calculated for MAP 2 content which was determined to be 75% of total protein (Vallee et al., 1981).

<sup>b</sup> Bio-Gel A-15m-purified MAP 2 was subjected to preparative gel electrophoresis before or after exposure to C. The excised MAP 2 gel chip was ashed for phosphate analysis.

<sup>c</sup> Heat-stable MAPs were further purified by passage over a column of Bio-Gel A-15m. Data for sample H are from the experiment shown in Figs. 2 and 3.

<sup>d</sup> These values were obtained from <sup>32</sup>P incorporation.
number of phosphates may be bound to MAP 2 prior to isolation.

Dephosphorylation of MAP 2—Using acid phosphatase pretreated with a variety of protease inhibitors (Collins and Korn, 1980) MAP 2 could be substantially dephosphorylated. However, proteolytic degradation was still extensive. We have found that inclusion of the protease inhibitor α2-macroglobulin reduced the extent of proteolysis considerably. Exposure of MAP 2 to acid phosphatase pretreated in this manner reduced bound phosphate to 1.4–3.3 mol/mol of MAP 2 (Table I, samples A–C, H, and I, column 6), while the MAP 2 polypeptide remained largely intact (Fig. 1). Analysis of the UV spectrum of material released from MAP 2 by exposure to phosphatase revealed less than 1 mol/mol of adenine nucleotide (data not shown). This indicates that most of the phosphate bound to MAP 2 is not present in this form (see Amir-Zaltsman et al., 1982).

cAMP-dependent phosphorylation of MAP 2—To determine the number of cAMP-dependent phosphorylation sites available on MAP 2, highly purified catalytic subunit prepared from the MAP 2-associated cAMP-dependent protein kinase (Vallee et al., 1981; Theurkauf and Vallee, 1982) was used. Phosphate incorporation into heat-stable MAPs was determined both for MAPs as isolated and after exposure to acid phosphatase.

Phosphorylation of heat-stable MAPs as isolated (Table I, samples A–C, column 4) resulted in the incorporation of an additional 6.5–13.0 mol of phosphate/mol of MAP 2, bringing total bound phosphate to 19.5–21.4 mol/mol of MAP 2. All of this phosphate appeared to be covalently linked to MAP 2 as indicated by analysis of electrophoretically purified MAP 2 (Table I, sample F, column 4). Phosphorylation of dephosphorylated MAP 2 increased the phosphate level to 10.5–14.7 mol/mol of MAP 2 (Table I, samples A–C, column 7). These values were substantially below those obtained with the control as isolated preparations (Table I, samples A–C, column 4). Nonetheless, the increase in phosphate content for both preparations was similar, with the highest values obtained being virtually identical (13.0–13.3 mol/mol).

To further compare as isolated with dephosphorylated MAP 2, phosphorylation catalyzed by purified cAMP-dependent protein kinase catalytic subunit was assayed by [32P]phosphate incorporation (Fig. 2). The time course of phospho-

![Figure 1. Electrophoretic analysis of MAP 2 preparations. A, heat-stable MAPs; B, MAP 2 purified by Bio-Gel A-15m chromatography of heat-stable MAPs. UN, preparations untreated with acid phosphatase; DE, preparations dephosphorylated with acid phosphatase prior to heat treatment. A minor extent of proteolysis was detectable in the heat-stable MAPs after exposure to phosphatase (arrows), but the observed fragments were eliminated during Bio-Gel A-15m purification.](http://www.jbc.org/)

![Figure 2. Time course of phosphorylation of MAP 2 by cAMP-dependent protein kinase. MAP 2 was purified by Bio-Gel A-15m chromatography with or without prior exposure to acid phosphatase (see Fig. 1B, untreated and dephosphorylated samples). The untreated sample contained 7.4 mol of phosphate/mol of protein, while the dephosphorylated sample contained 3.1 mol of phosphate/mol of protein. The MAP 2 (1 mg/ml) was exposed to the catalytic subunit of cAMP-dependent protein kinase (20 μg/ml) in the presence of 500 μM [32P]ATP, and [32P]incorporation into MAP 2 was determined at a series of times. ○, untreated MAP 2; ●, dephosphorylated MAP 2.](http://www.jbc.org/)
ylation was identical for as isolated MAP 2 and dephosphorylated MAP 2. The final extent of phosphate incorporation (~10 mol/mol) was comparable to that determined by inorganic phosphate analysis (Table I) and was identical for both MAP 2 preparations despite a 4.3 mol/mol difference in initial phosphate content. The distribution of phosphates in the MAP 2 polypeptide chain was evaluated by peptide mapping (Fig. 3) for the two preparations shown in Fig. 2. The pattern of phosphoproteins for the two preparations was identical in this experiment, indicating that the same CAMP-dependent phosphorylation sites were available in both MAP 2 as isolated and following dephosphorylation.

On the basis of our results, we propose that MAP 2 contains as many as 13 CAMP-dependent phosphorylation sites, the maximum extent of phosphate incorporated with CAMP-dependent protein kinase catalytic subunit, using either MAP 2 as isolated or dephosphorylated MAP 2. We estimate that as many as 8 mol of phosphate are present in MAP 2 as isolated that cannot be accounted for by CAMP-dependent phosphorylation. We suggest that these phosphates are introduced into MAP 2 by other enzymes, the identity of which are at present unknown. One candidate for such an enzyme is a protein kinase associated with neurofilaments, which has been found to be capable of phosphorylating MAP 2 (Runge et al., 1981). Whether this kinase or others as yet unidentified can account for the non-CAMP-dependent phosphates in MAP 2 remains to be determined.

Alternatively, it is possible that all of the MAP 2 phosphates can be introduced by CAMP-dependent protein kinase, but a fraction of the sites (about eight) are not readily filled. To account for the low values previously reported for incorporation of [32P]phosphate into MAP 2 (Sloboda et al., 1981), we suggest that MAP 2 was in an almost fully phosphorylated state in these studies. We do not understand why higher levels of phosphate incorporation into MAP 2 were not observed in an earlier phosphate exchange study (Coughlin et al., 1980), although a high level of phosphatase activity in the microtubule preparation that was used could have depressed the steady state level of phosphate incorporation. High levels of phosphate incorporation into MAP 2 have been reported in one study (Islam Burns, 1981), although the identity of the kinase involved was unknown. Only ~7 mol of phosphate were incorporated per mol of MAP 2, apparently due to a phosphatase activity which is eliminated from our preparations during microtubule purification (Vallee et al., 1981). Nonetheless, the data were extrapolated to values as high as 10–12 mol of phosphate/mol of MAP 2, consistent with the present study.

In conclusion, it now appears that MAP 2 contains numerous sites for phosphorylation. In view of the concentration of MAP 2 in brain tissue (~1% soluble protein; Valdivia et al., 1982) and the numerous CAMP-dependent phosphorylation sites found in the present study, MAP 2 must be a major substrate for CAMP-dependent phosphorylation in brain. In fact, we find MAP 2 to be the major substrate for endogenous CAMP-dependent phosphorylation in unfractionated cytosolic extracts of brain tissue (data not shown). In particular, in view of the predominant localization of MAP 2 in neurons and its preferential localization in the dendritic processes of these cells (Matus et al., 1981; Miller et al., 1982; Vallee, 1982), MAP 2 phosphorylation may be an important reaction occurring in response to neurotransmitter stimulation.

REFERENCES


Extensive cAMP-dependent and cAMP-independent phosphorylation of microtubule-associated protein 2.

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