Phosphorylation Determines the Binding of Microtubule-associated Protein 2 (MAP2) to Microtubules in Living Cells

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Abstract. The influence of phosphorylation on the binding of microtubule-associated protein 2 (MAP2) to cellular microtubules was studied by microinjecting MAP2 in various phosphorylation states into rat-1 fibroblasts, which lack endogenous MAP2. Conventionally prepared brain MAP2, containing 10 mol of endogenous phosphate per mol (MAP2-P₁₀), was completely bound to cellular microtubules within 2-3 min after injection. MAP2 prepared in the presence of phosphatase inhibitors, containing 25 mol/mol of phosphate (MAP2-P₂₅), also bound completely. However, MAP2 whose phosphate content had been reduced to 2 mol phosphate per mol by treatment with alkaline phosphatase in vitro (MAP2-P₂) did not initially bind to microtubules, suggesting that phosphorylation of certain sites in MAP2 is essential for binding to microtubules. MAP2-P₁₀ was further phosphorylated in vitro via an endogenously bound protein kinase activity, adding 12 more phosphates, giving a total of 22 mol/mol. This preparation (MAP2-P₁₀+₁₂) also did not bind to microtubules. Assay of the binding of these preparations to taxol-stabilized tubulin polymers in vitro confirmed that their binding to tubulin depended on the state of phosphorylation, but the results obtained in microinjection experiments differed in some cases from in vitro binding. The results suggest that the site of phosphate incorporation rather than the amount is the critical factor in determining microtubule binding activity of MAP2. Furthermore, the interaction of MAP2 with cellular microtubules may be influenced by additional factors that are not evident in vitro.

MAP2 and then localize the MAP2 by immunofluorescence. In two studies of this type, all the injected MAP2 was found to bind to the cellular microtubules (Scherson et al., 1984; Olmsted et al., 1989). In contrast, an immunocytochemical study of endogenous MAP2 distribution in cultured primary neurons showed that it was not colocalized with tubulin polymers but was distributed throughout the cytoplasm (Matus et al., 1986).

A potential explanation of these differences lies in the phosphorylation state of native MAP2 in nerve cells growing in culture compared to biochemically isolated MAP2 preparations used in microinjection experiments. Available data suggests that the phosphate content of MAP2 decreases significantly during its isolation from brain (Tsuyama et al., 1987). Our experiments were therefore designed to test the influence of phosphorylation state of MAP2 on its binding to microtubules in living cells. We compared MAP2 in four states of phosphorylation: (a) as obtained from brain; (b) as obtained from brain in the presence of phosphatase inhibitors; (c) after enzymatic dephosphorylation in vitro; and (d) after additional in vitro phosphorylation. MAP2 in each of these phosphorylation states was purified chromatographically then injected into fibroblastic cells, and the relationship of the MAP2 to the cellular microtubules was determined by

1. Abbreviation used in this paper: MAP2, microtubule-associated protein 2.
immunofluorescence staining. These experiments reveal that
these four distinct phosphorylation states significantly in-
fluence the interaction of MAP2 with cellular microtubules.

Materials and Methods

Cell Culture

Rat-1 fibroblasts were cultured in DME (GIBCO, Renfrewshire, Scotland) supplemented with 10% FCS. For some experiments "serum-starved" cells were maintained in the same medium without serum for 12 h before begin-
ning the experiment.

Preparation of MAP2

MAP2 was prepared either from repolymerized microtubules or directly from brain supernatant. Microtubules were prepared from adult rat brain by three cycles of repolymerization as described by Karr et al. (1979). To obtain microtubules with minimal dephosphorylation of MAPs, brains were removed from individual animals and immediately homogenized in micro-
tube assembly buffer (0.1 M MES, pH 6.4, 2 mM EGTA, and 1 mM MgCl2) containing the phosphatase inhibitors sodium fluoride (20 mM) and sodium pyrophosphate (10 mM). The homogenates were collected at ice temperature and then centrifuged for 45 min at 4°C to 100,000 g. To obtain heat-stable MAPs from either recycled microtubules or brain superna-
tant, the preparations were made up to 0.75 M NaCl and 2 mM DTT, boiled for 5 min, and then centrifuged at 100,000 g for 60 min (Fellous et al., 1977). These preparations were concentrated to 10 mg protein per ml using Centrifloc 30 microconcentrators (Amicon, Zürich) and the MAPs were fractionated by gel filtration chromatography on a Superose 6 column (Pharmacia, Dünbendorf) in microtubule assembly buffer containing 0.3 M KCl. The MAP content of fractions was assayed by immobilizing 0.5 μl dots on nitrocellulose and staining for MAP2, MAP3, tau, and tubulin with mAbs. The major MAP2 peak was collected and concentrated using Centri-
loc 30 ultracentrifugation. The purified proteins at 5 mg/ml were stored as aliquots at –70°C until required for microinjection.

Enzymatic Dephosphorylation of MAP

Heat-stable MAPs (5 mg/ml) were treated with calf intestinal phosphatase in buffer containing 0.1 M Tris HCl, pH 8.4, 1 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 2 mM PMSF, 1 μg/ml pepstatin, 1 U/ml aprotinin, and 0.1 mM ZnCl2 for 12 h at 37°C (Tsuyama et al., 1986; Selden and Pollard, 1983). From a range of commercial preparations tested, only that from Boehringer (Mannheim) (cat. # 713-023, 25 U/ml) combined good phosphatase and protease activity toward MAPs. Nevertheless, protease inhibitors were used in all experiments. To achieve maximal dephosphorylation, fresh phosphatase was added to the reaction every 3 h to a final concentration of 100 U/ml. The phosphatase was subsequent-
ly inactivated by heating for 2 min in a boiling water bath.

In Vitro Phosphorylation of MAP

MAP2 was phosphorylated via endogenous kinase activity essen-
tially as described by Burns et al. (1984). Microtubule proteins (5 mg/ml) were phosphorylated in a buffer containing 0.1 M MES, 20 mM sodium fluoride, 2.5 mM EGTA, 0.5 mM MgSO4, 0.1 mM EDTA, 0.1 mM DTT, 20 μM GTP, and 500 μM ATP for 30 min at 37°C. The reaction was stopped by adding NaCl to 0.75 M and heating in a boiling water bath for 5 min. The phosphate contents of purified MAP2 samples were determined according to Selden and Pollard (1983).

Gel Electrophoresis and Immunoblotting

4-14% linear gradient SDS-polyacrylamide electrophoretic gel were run ac-
cording to Laemmli (1970). Proteins were transferred to nitrocellulose elec-
trophoretically (Towbin et al., 1979), visualized by staining with 2% Ponceau red in 3% perchorlic acid, and destaining with 10% acetic acid. The stain was washed out with 100 mM PBS (150 mM NaCl, pH 7.2) and the blots were immunostained using mAbs (Towbin et al., 1979).

Microinjection Procedure

Purified MAP2 was microinjected into rat-1 cells in assembly buffer con-
taining 0.3 M KCl (Scherson et al., 1984) through glass capillary needles at constant pressure. The cells were plated at a density of 2 × 10⁴ cells/cm² in 35-mm tissue culture dishes containing a finder grid pattern and grown for 24 h. During microinjection the culture medium contained 10 mM Hepes buffer, pH 7.5, and the culture dish was maintained at 37 ± 0.5°C using an aluminum block thermostat fixed on the microscope stage. The amount of MAP2 injected was assessed visually using the phase-
contrast optics of a Zeiss IM-35 inverted microscope (Zeiss, Zürich) at x250 magnification and was controlled by the length of time that the tip remained inside the cell. The injected volume varied from 1 to 10% of the cell volume. In each experiment cells were injected at 30-s intervals, so that at the time of fixation a series of cells representing a 0-30-min time course of postinjection survival were present in the same dish.

Immunofluorescent Labeling of Cultured Cells

Cells were rinsed with PBS, fixed with 3% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized by treatment with 0.05% Triton X-100 in PBS for 10 min. Nonspecific binding sites were blocked by treatment for 10 min with 5% FCS in PBS, and the cells then incubated overnight with a 1:50 dilution of rabbit antiserum against tubulin (Poly-
sience, Ulmenhof, West Germany) and AP14 mAb against MAP2 (Ca-
ceres et al., 1984) at 4°C. After washing, the cells were incubated for 2 h at room temperature with fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled goat antimouse (Nordic, Biogenzia Lemania, Lausanne) each diluted 1:200 and observed in a Zeiss Universal fluorescence micro-
scope.

In Vitro Binding Assay

The binding of MAP2 in various states of phosphorylation to taxol-
stabilized tubulin polymers was assayed as described by Burns et al. (1984). Tubulin was purified from recycled microtubules as described by Hannel and Lin (1981) and concentrated in assembly buffer (Karr et al., 1979) using Centrinoc 30 microconcentrators. Samples consisted of purified MAP2 and tubulin in a molar ratio of 1:12. They were brought to various concentrations of NaCl as required, incubated with taxol (20 μM; Vallee, 1982) for 15 min at 37°C, and pelleted in an Airfuge (Beckman, Geneva). The pellets were resuspended in sample buffer and separated by electrophoresis in 7% SDS-polyacrylamide gels (Laemmli et al., 1970). These were stained with Coomasie brilliant blue and the relative protein contents of the bands were determined as peak heights measured in a Shimadzu reflectance scanner (Burkhardt Instruments, Zürich). To account for minor varia-
tions in the amount of tubulin the MAP2 measurements for each sample were normalized using the peak heights of the tubulin band taken from the same scan.

Results

Preparation of MAP2 with Different Levels of Bound Phosphate

To distinguish the four different phosphorylation states of MAP2 studied in these experiments, we used a designation of the type MAP-Pn, where the subscript number indicates the average number of bound phosphates per mol of MAP2 in that particular preparation. MAP2 prepared from conventional recycled microtubule preparations or directly from rat brain supernatant (SI) in absence of phosphatase inhibitors has about 10 covalently bound phosphates per molecule (MAP2-P10; Table I). This represents only part of the phosphate content of the native molecule because MAP2 is rapidly dephosphorylated during this conventional preparation procedure (our unpublished observations, see also Murthy and Flavin, 1983; Tsuyama et al., 1987). To obtain MAP2 with more of the endogenous phosphate intact, we isolated it directly from brain superna-
tant in presence of phosphatase inhibitors and this, in conjunc-
tion with rapid processing during the initial steps, yielded MAP2 with a phosphate level of ~25 mol of covalently bound phosphate per mol (MAP2-P25; Table I).
assess further the role of phosphorylation, we dephosphorylated MAP2-P10 enzymatically. Maximal dephosphorylation (see Materials and Methods) reduced the MAP2 phosphate content to 2 mol/mol (MAP2-P2; Table I). Finally, we also conducted experiments with MAP2 that had been phosphorylated in vitro via a copurifying kinase activity (Vallee et al., 1981; Burns et al., 1984). This resulted in the additional incorporation of up to 12 phosphates into MAP2-P10, yielding MAP2 with 22 mol of covalently bound phosphate (MAP2-P10+12; Table I).

MAP2 in these various phosphorylation states was purified from the mixture of heat-stable MAPs by gel filtration chromatography and the MAP2-containing fractions were identified and their purity analyzed by immunodot assay (Fig. 1 A), after which the MAP2 fractions were collected, concentrated, and their purity and freedom from degradation confirmed by SDS-PAGE (Fig. 1 B).

**Microinjection Experiments**

Rat-1 fibroblast cells have two advantages as recipient cells for MAP2 microinjection. First, as is evident from noninjected cells in Figs. 2-6, they do not express detectable levels of MAP2, a necessary condition if microinjected MAP2 is to be unambiguously identified. Secondly, these cells possess only a moderate density of cytoplasmic microtubules whose distribution can be resolved clearly by immunofluorescence staining with antitubulin. The results described are based on observations of several hundred cells from more than 50 individual cultures into which samples from 3 different preparations of purified MAP2 in each phosphorylation state were microinjected. The same results were obtained in all cases.

**Intracellular Distribution of Microinjected MAP2**

MAP2 in Either of Two Endogenous Phosphorylation States Binds to Cellular Microtubules. When MAP2-P10 (i.e., conventionally prepared MAP2) was injected into Rat-1 cells it initially diffused throughout the cytoplasm (Figs. 2b and 3b). However, by 3 min after injection it had started to bind to microtubules (Figs. 2d and 3d), and by 5 min

![Figure 1](image-url)

**Figure 1.** MAP2 purification. Heat-stable MAPs were fractionated on a Superose 6 column, and the contents of each fraction was established by immunodot assay (A) using mAbs against MAP2, MAP3, Tau, and Tubulin (TU). The MAP2 fractions were collected, concentrated, and analyzed by SDS-PAGE (B): (lane A) supernatant fraction from brain; (lane B) thrice-cycled brain microtubules; (lane C) heat-stable brain MAPs; (lane D) purified MAP2.
Figure 2. Intracellular localization of tubulin and microinjected MAP2 prepared in absence of phosphatase inhibitors (MAP2-P0) in rat-1 cells. Tubulin (a, c, and e) and MAP2 (b, d, and f) were simultaneously detected in the same cell cultures by staining with mouse monoclonal anti-MAP and rabbit polyclonal antitubulin followed by rhodamine-labeled antimouse and fluorescein-labeled anti-rabbit IgGs. In this figure and Figs. 3–6, microinjected cells are identified in the antitubulin-stained images by asterisks. MAP2 is not bound to microtubules after 2 min (a and b), is partially bound after 3–4 min, binds to microtubules (c and d), and decorates all the microtubules present in the cell after 15 min (e and f). Bar, 10 µm.

After injection all the detectable MAP2 was associated with microtubules and this continued to be the case until 30 min after injection, the longest postinjection time tested. Figs. 3 f and 4 f show this at 15 min postinjection. The same results were obtained when MAP2 prepared in the presence of phosphatase inhibitors (MAP2-P25) was injected (data not shown).
Enzymatically Dephosphorylated MAP2 Does Not Bind to Microtubules. Each batch of dephosphorylated MAP2 (MAP2-P_2) was examined by SDS-PAGE to confirm that it had not been degraded by treatment with alkaline phosphatase. As a further control in all dephosphorylation experiments, an additional sample of the starting preparations (MAP2-P_0) was incubated in the same buffer and under the same conditions, but without the addition of enzyme. This control MAP2 was microinjected into Rat-1 cells and it was confirmed that it bound to cellular microtubules. The dephosphorylated MAP2 (MAP2-P_2) showed a different pattern of behavior. Like the control MAP2-P_0 it was initially distributed throughout the cytoplasm after injection (Fig. 4 b). However, with MAP2-P_2 this condition persisted longer than in the previous cases and after 4 min it still showed no binding to microtubules (Fig. 4 d). Only after 15 min did some binding of injected cells MAP2-P_2 to microtubules in the periphery of the cell become visible, but large amounts still remained spread throughout the cytoplasm (Fig. 4 f).

We considered the possibility that this delayed binding of dephosphorylated MAP2 to microtubules might reflect a slow rephosphorylation by cellular protein kinases of certain sites on the molecule that are essential for microtubule binding. To test this hypothesis we injected dephosphorylated MAP2 into cells that had been serum starved for 24 h when protein kinase activities and MAP phosphorylation levels are lower (Shaw et al., 1988). The result of this experiment was that the injected MAP2-P_2 remained spread throughout the cytoplasm and did not bind to microtubules even after 30 min (Fig. 5 b). To eliminate the possibility that this lack of binding might be the result of some change in the microtubules (i.e., the tubulin polymers) induced by the serum-free conditions, we injected highly phosphorylated MAP2-P_5 into the serum-starved cells. In this case the MAP2-P_5 bound normally to the microtubules (Fig. 5 d).

In Vivo and In Vitro Phosphorylation of MAP2 Have Different Effects. The above experiments indicated that phosphorylation of MAP2 promotes its binding to microtubules. This result is at first sight contradictory to previous studies in which the phosphorylation of MAP2 was found to decrease both its tubulin polymerization promoting activity (Jameson and Caplow, 1981) and its affinity for microtubules (Burns et al., 1984). However, in these earlier experiments the phosphorylation of MAP2 was carried out in vitro using endogenous kinase activity that copurifies with MAP2 (Sloboda et al., 1975; Vallet et al., 1981). This suggested to us that there might be two different states of MAP2 phosphorylation, an endogenous neuronal state that, as the above experiments show, is necessary for MAP2 binding to microtubules and a second, in vitro phosphorylation state, that lowers the affinity of MAP2 for microtubules. To test this hypothesis we took MAP2 prepared by conventional means (MAP2-P_0) and phosphorylated it in vitro via the bound kinase activity. This introduced 12 extra bound phosphates into the molecule (MAP2-P_12, Table I). When injected into cells, this in vitro phosphorylated MAP2 did not bind to microtubules even after 30 min (Fig. 6). As a control, MAP2-P_0 was incubated in the same buffer under the same conditions, but without the addition of ATP. When this control preparation was injected into cells it bound to microtubules.

In Vitro Binding of MAP2 in Different Phosphorylation States to Taxol-stabilized Microtubules. Fig. 7 shows assays of the in vitro tubulin-binding capacity of MAP2 in each of the four phosphorylation states. In the presence of increasing salt concentrations the preparations bound to taxol-stabilized microtubules with different affinities that broadly reflected their binding to cellular microtubules, with the two preparations that do not bind to cellular microtubules (MAP2-P_2 and MAP2-P_5) binding with the lowest affinities.
Figure 4. Dephosphorylated MAP2 (MAP2-P2) shows reduced binding to microtubules. MAP2-P2 does not bind to microtubules 2 or 4 min after microinjection (b and d). Only after 15 min is any colocalization with tubulin filaments detectable (e and f), and even then a substantial portion of MAP2-P2 remains cytosolic (e and f). Bar, 10 μm.

An interesting feature of the results is that although the binding performance of MAP2-P10 and MAP2-P10+12 in vitro was not very different, in microinjection experiments the former appears completely bound to cellular microtubules, but the latter not at all, suggesting that in living cells phosphorylation of MAP2 has a greater effect on its binding to microtubules than can be detected in vitro. It is also worth
mentioning that we saw a small but consistent difference in the elution of MAP2-P25 and MAP2-P10 from taxol-stabilized microtubules, even though we observed no difference in their binding to cellular microtubules after microinjection.

**Discussion**

*Different Phosphorylation Sites in MAP2 Have Distinct Effects on Its Interaction with Cellular Microtubules*

Our results show that MAP2 can exist in at least four distinct states of phosphorylation that differently influence its binding to microtubules in living cells. Two of these, with phosphate contents of 25 or 10 mol/mol, are endogenously phosphorylated states that bind effectively to cellular microtubules. The other two, enzymatically dephosphorylated MAP2 containing 2 mol/mol of phosphate, and in vitro phosphorylated MAP2 containing 22 mol/mol of phosphate, show no appreciable binding to cellular microtubules.

The lack of binding to cellular microtubules by MAP2 after dephosphorylation is correlated with a reduction in its binding to taxol-stabilized microtubules in vitro. The fact that it retains some in vitro binding activity indicates that its failure to bind to microtubules in cells is not merely the result of denaturation caused by the enzyme treatment. Further evidence in favor of this interpretation is that when dephosphorylated MAP2 is injected into actively growing cells it slowly regains microtubule binding capacity. The reappearance of microtubule binding activity suggests that the dephosphorylated MAP2 undergoes rephosphorylation by protein kinases in the recipient fibroblast cells. This interpretation is supported by the observation that the reemergence of microtubule binding is not seen in quiescent serum-starved cells, where kinase activity is low (Shaw et al., 1988). Thus, one or more of the 10 mol of endogenous bound phosphates present in conventionally prepared MAP2 appear to be essential for its binding to microtubules.

MAP2 phosphorylated in vitro via its endogenously bound kinase activity (Sloboda et al., 1975; Vallee et al., 1981) also shows reduced affinity for microtubules in vitro (Murthy and Flavin, 1983; Burns et al., 1984; this study), which correlates with its lack of binding to microtubules in living cells. It is striking that MAP2 phosphorylated in vitro contains 22 mol/mol of phosphate and does not bind to cellular microtubules, whereas MAP2 purified from brain in the presence of phosphatase inhibitors contains more phosphate (25 mol/mol) and binds strongly. Also the difference in microtubule
binding affinity of these two forms in vitro, while distinct, is not great. These observations indicate that it is not the level of phosphorylation that is important in determining binding of MAP2 to microtubules in cells, but some other quality of the phosphorylated state, most likely the location of the phosphorylated sites within the molecule.

There is very little data on the precise location of endogenous phosphorylation sites in MAP2. Both cAMP-dependent and Ca\(^{2+}\)/calmodulin kinases have been shown to phosphorylate sites in the portion of the molecule that is bound to microtubules (Vallee, 1980; Schulman, 1984; Vallano et al., 1985; Hernandez et al., 1987). However, it is clear that the degree of phosphorylation of all in vitro preparations is less than that of MAP2 in the living brain (Tsuyama et al., 1987), and other phosphorylation sites might exist that also influence the interaction of MAP2 with microtubules. This possibility is suggested by experiments showing that various well-characterized kinases phosphorylate different sites on MAP2 in vitro (Hernandez et al., 1987) and that at least some MAP2 sites endogenously phosphorylated in the brain differ from those phosphorylated by identified kinases in vitro (Murthy et al., 1985).

**Phosphorylation of MAP2 May Be Required for Microtubule Binding**

The microtubule binding site of MAP2 has been localized to...
a region near the COOH-terminus of the molecule containing 3 imperfectly repeated 18 amino acid sequences (Lewis et al., 1988). Synthetic oligopeptides based on the second of these sequences, m2 (Joly et al., 1989), can bind to tubulin in vitro, showing that phosphorylation is not necessary for the interaction between the MAP2 binding site and tubulin. Why then, in our experiments, was dephosphorylation correlated with a decrease in MAP2 affinity for microtubules both in vitro and in cells? The answer is probably that our experiments used the whole MAP2 molecule, of which the m2 sequence represents only 18 amino acids out of 1,828. Other parts of the MAP2 molecule may influence the affinity of the binding domain, for example, by folding in such a way as to occlude it, and this folding may in turn be influenced by phosphorylation at sites in the molecule remote from the microtubule-binding domain.

**The Potential Influence of MAP2 Phosphorylation on Neuronal Morphology**

In addition to stabilizing tubulin polymers, MAP2 is also a component of fine filamentous cross-bridges between neuronal microtubules (Hirokawa et al., 1988). Transfection experiments, in which MAP2 was expressed in nonneuronal cells from cloned cDNA, show that it can induce the bundling of microtubules (Lewis et al., 1989). Changes in the affinity of MAP2 for tubulin, resulting from changes in the phosphorylation state, would therefore influence not only the stability of microtubules, but also the fasciculation of dendritic microtubules, which is essential for the maintenance of the axial symmetry of neuronal processes (Matus, 1988).

Aoki and Siekevitz (1985) found that a marked change occurs in MAP2 phosphorylation at the end of the critical period in the cat visual cortex. A compelling aspect of their results is that when the critical period was prolonged by raising animals in the dark, the change in MAP2 phosphorylation was delayed in parallel. Furthermore, both the end of the critical period and the change in phosphorylation could be induced by a short exposure of the dark-reared animals to light. Recently, Halpain and Greengard (1989) have shown that stimulation of NMDA-class glutamate receptors in brain slices induces net dephosphorylation of MAP2. Taken together with our results, these data suggest that changes in MAP2 phosphorylation, triggered by sensory inputs to the nervous system, may influence the stability of the microtubular network and hence regulate the anatomical plasticity of neuronal dendrites in the developing brain.

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