

Heterogeneity of microtubule-associated protein 2 during rat brain development

(microtubules/monoclonal antibodies/immunoblotting/phosphorylation)

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ABSTRACT The electrophoretic pattern of the large microtubule-associated protein, MAP2, changes during rat brain development. Immunoblots of NaDodSO₄ extracts obtained from the cerebral cortex, cerebellum, and thalamus at 10–15 days after birth reveal only a single electrophoretic species when probed with any of three MAP2 monoclonal antibodies. By contrast, adult MAP2 contains two immunoreactive species, MAP2a and MAP2b. The single band of MAP2 from immature brain electrophoretically comigrates with adult MAP2b. Between postnatal days 17 and 18, immature MAP2 simultaneously resolves into two species in both the cerebellum and cerebral cortex. Immunoblots of NaDodSO₄ extracts from spinal cord demonstrate the adult complement of MAP2 by day 10, indicating that MAP2 does not change coordinately throughout the entire central nervous system. *In vitro* cAMP-dependent phosphorylation of immature MAP2 causes a band split reminiscent of that seen during brain development *in vivo*. The possibility that the developmentally regulated changes observed in MAP2 during brain maturation are due to timed phosphorylation events is discussed.

Microtubule-associated protein 2 (MAP2) is a heat-stable phosphoprotein (M_r 300,000) that copurifies with brain microtubules *in vitro* and maintains a constant stoichiometry to tubulin through several cycles of temperature-dependent assembly and disassembly (1, 2). It is one of a number of high molecular weight polypeptides associated with *in vitro* assembled brain microtubules, collectively referred to as HMW MAPs (3). NaDodSO₄/urea/polyacrylamide gel electrophoresis separates MAP2 into two closely migrating polypeptides, which we designate MAP2a and MAP2b for the slower and faster migrating species, respectively. *In vitro*, MAP2 will stimulate the assembly of purified tubulin, lowering the critical concentration for tubulin assembly (1). When such preparations are visualized with the electron microscope, MAP2 appears as regularly spaced filamentous sidearms decorating the surface of microtubules (1), reminiscent of the appearance of microtubules *in situ* (4, 5). In addition to its interaction with microtubules, MAP2 interacts with neurofilaments and actin filaments (3, 6–10). This suggests that MAP2 may act as a crosslinker mediating the interaction between microtubules, other cytoskeletal elements, and cytoplasmic organelles. Evidence is accumulating which suggests that the relative affinity of MAP2 for different cytoskeletal structures may be controlled by its degree of phosphorylation (10–12).

Radioimmunoassay of a variety of tissues suggests that only brain contains significant amounts of MAP2 (13). Fluorescence, light, and immunoelectron microscopic studies using monoclonal antibodies have localized MAP2 in neurons

but not glia (13, 14). Biochemical and morphological studies further indicate that within nerve cells more of this protein is present in dendrites and cell bodies than in axons (14–16). Furthermore, MAP2 from neonatal rat brain was shown to be biochemically distinct from its adult counterpart in that it was less efficient in stimulating the rate of microtubule assembly *in vitro* (17).

To further understand the role of proteins associated with brain microtubules, we examined MAP2 during brain development. Using monoclonal antibodies specific for the MAP2 doublet (MAP2a and MAP2b), we discovered that in both NaDodSO₄ extracts and in taxol-stabilized microtubules prepared from different areas of the central nervous system (CNS), MAP2 changes its electrophoretic pattern during development. At postnatal day 10, MAP2 is a single band that coelectrophoreses with adult MAP2b (the faster migrating polypeptide species), whereas by 17–18 days, the mobility of MAP2 is altered to the double-banded adult form. This change occurs simultaneously in the cerebellum, cerebral cortex, and thalamus but already has occurred by day 10 in the spinal cord. Phosphorylation of MAP2 from 10-day microtubules assembled *in vitro* causes a polypeptide doublet to appear, mimicking the change observed in the NaDodSO₄ extracts during development.

MATERIALS AND METHODS

Rat brain tissue was obtained from Sprague–Dawley adults and pups at the postnatal ages indicated. Freshly dissected brain regions were placed directly into a Dounce homogenizer containing 0.0625 M Tris-HCl, pH 6.8/2% NaDodSO₄/10% glycerol/5% 2-mercaptoethanol (18) and homogenized in a boiling water bath for 4 min. After sedimentation at 25°C for 1 hr at 100,000 × *g*, the supernatant was removed and either used immediately for electrophoresis or stored at –80°C.

NaDodSO₄/urea/polyacrylamide gradient slab gels were run by the method of Laemmli (18) as modified by Binder and Rosenbaum (19). The separating gels were composed of 1–8 M linear urea gradients superimposed on 3–12% linear acrylamide gradients. Gels were stained with Coomassie blue R or were transferred to nitrocellulose sheets for subsequent amido black or immunostaining (20). Antibody binding was visualized by using a second antibody-peroxidase conjugate and appropriate substrate (20).

Taxol-stabilized microtubules were made and isolated from various areas of the brain by the method of Vallee (16). Phosphorylation was performed on taxol-stabilized microtubules by the method of Sloboda *et al.* (2) as modified by Theurkauf and Vallee (12) by utilizing the endogenous,

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Abbreviations: MAP, microtubule-associated protein; CNS, central nervous system.

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MAP2-associated cAMP-dependent protein kinase (2, 12, 21). The reaction was stopped by adding electrophoresis sample buffer and boiling for 3 min.

Two monoclonal antibodies (AP-7 and AP-13) to different epitopes on MAP2 were used in these experiments. Both of the antibodies are IgGs, and their production, selection, and characterization have been reported elsewhere (14, 22, 23).

RESULTS

Electrophoretic Changes in MAP2 in NaDodSO₄ Extracts of Whole Tissue. Since cerebellar development is predominantly postnatal, this structure was the first assayed for potential alterations in MAP2. At postnatal day 10, immunostains of NaDodSO₄ extracts showed reactivity with only a single electrophoretic species of MAP2 (Fig. 1). In contrast, extracts of adult cerebella exhibited the classic MAP2 doublet (Fig. 1), MAP2a and MAP2b.

Other regions of the rat CNS were then assayed for MAP2 heterogeneity at 10 days after birth. Specifically, the cerebral cortex, the thalamus, and the spinal cord were extracted, blotted, and stained with monoclonal antibodies to MAP2. In both the cortex and the thalamus, MAP2 was a single band at day 10 (Fig. 2, lanes B and C). In contrast, however, the spinal cord contained a MAP2 doublet at this age and, like adult spinal cord (data not shown), displayed a minor immunoreactive species, MAP2c, which migrated more slowly than MAP2a (Fig. 2, lane D). Other experiments demonstrated that spinal cord MAP2 contained a single immunoreactive species at postnatal day 2 (data not shown). Therefore, a change in spinal cord MAP2 occurred significantly earlier than the MAP2 alterations observed in the other brain regions.

To determine when the adult form of MAP2 appeared in the cortex and cerebellum, rat pups were sacrificed at postnatal days 13, 15, 17, 18, and 25. Subsequent blotting and immunostaining with MAP2 monoclonal antibodies indicated that the first doublet form of MAP2 was present between 17 and 18 days after birth in both the cerebellum (Fig. 3A) and the cerebral cortex (Fig. 3B), with the adult separation firmly established between postnatal days 18 and 25. Similar results were also obtained regarding the MAP2 change in the thalamus (data not shown).

MAP2 Changes Observed in Taxol-Stabilized Microtubules. Whether MAP2 from immature brains was electrophoretically unique or similar to either MAP2a or MAP2b from adult animals was determined by using a comigration/mixing ex-

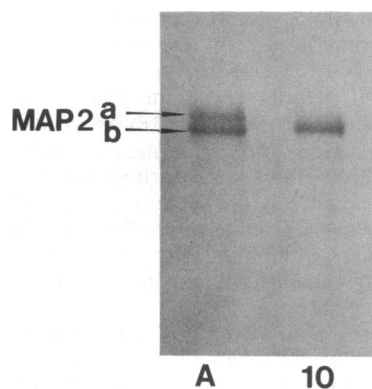


FIG. 1. Immunoblot of the high molecular weight region of an NaDodSO₄/urea gel. NaDodSO₄ extracts of whole cerebellum from adult (lane A) and postnatal day 10 (lane 10) rats were electrophoresed, transferred to nitrocellulose, and stained with the anti-MAP2 monoclonal antibody AP-13. The adult cerebellum contains two immunoreactive species, MAP2a and 2b, whereas the sample from 10-day cerebellum exhibited only one.

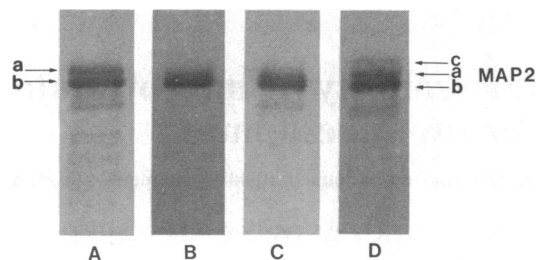


FIG. 2. Immunoblots of NaDodSO₄ extracts from adult cerebral cortex (lane A) and 10-day old cerebral cortex (lane B), thalamus (lane C), and spinal cord (lane D). The minor immunoreactive species migrating faster than MAP2b that are most evident in the adult cortex and 10-day-old spinal cord are proteolytic fragments of MAP2.

periment. Taxol-stabilized microtubules were prepared from both 10-day-old and adult rat brains and electrophoresed on the same slab gel. As expected, MAP2 in 10-day microtubules was present as a single band, whereas in adult microtubules, MAP2 appeared as a doublet (Fig. 4). Because of the numerous HMW MAP polypeptides present in both microtubule preparations (see Fig. 4), immunoblotting was performed and the MAP2 bands were visualized with a MAP2 monoclonal antibody. It is readily apparent (Fig. 5) that the single band of MAP2 present in 10-day-old rat brain coelectrophoresed with adult MAP2b.

In addition, when compared to adult preparations, taxol-stabilized microtubules from 10-day-old rat brains displayed other differences in HMW MAPs besides that documented for MAP2. Most notably, a HMW MAP doublet ($>$ MAP2 but $<$ MAP1) was in evidence in microtubule preparations from 10-day-old animals. We have designated these proteins MAPX (Fig. 4). Little MAPX was present in taxol-stabilized microtubules from adult animals. However, a polypeptide migrating slower than MAP2a but faster than MAPX was

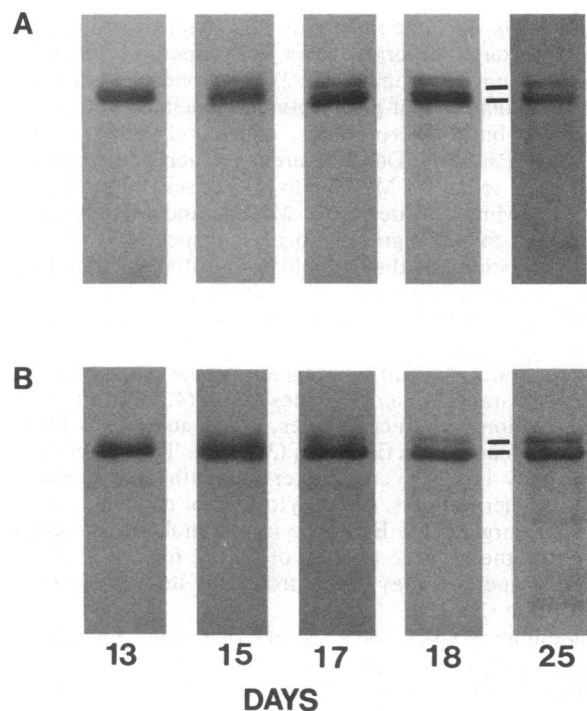


FIG. 3. Change in MAP2 during postnatal development of the cerebellum (A) and cerebral cortex (B). All are immunoblots of NaDodSO₄ extracts taken at the postnatal day indicated, and all were immunostained with the same MAP2 monoclonal antibody (AP-7).

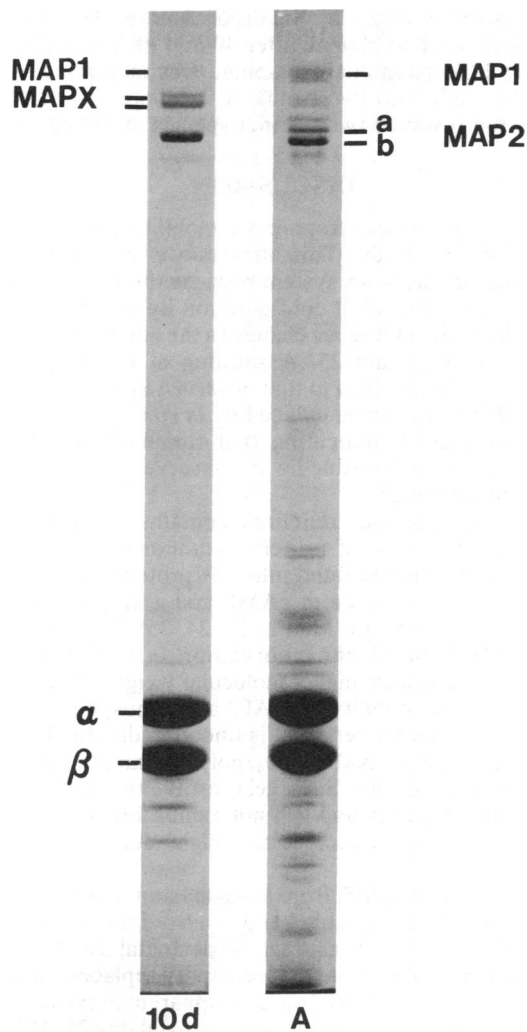


FIG. 4. NaDodSO₄/urea/polyacrylamide gels of taxol-stabilized microtubules made from 10-day-old (lane 10d) and adult (lane A) rat brains. MAP2 is a single electrophoretic species in taxol-stabilized microtubules from 10-day-old animals. In addition, a novel polypeptide doublet, MAPX, was evident in 10-day-old microtubules but less apparent in adult microtubules. The positions of MAP1 and α - and β -tubulin are also designated.

present in significant amounts in adult microtubule preparations (Fig. 4). Furthermore, less MAP1, the largest HMW MAP, was present in 10-day microtubules when compared to adult taxol-stabilized microtubules (Fig. 4). This electrophoretic pattern was similar to that obtained from twice-cycled microtubule preparations showing that taxol-stabilization does not result in the selection of different MAP species (data not shown).

Electrophoretic Changes in MAP2 Induced by *in Vitro* Phosphorylation. The coelectrophoresis experiment suggested that a single species of MAP2 identical to adult MAP2b was present in the immature cerebellum, cortex, and thalamus. However, the staging of the MAP2 change (Fig. 3) indicated that, rather than the immediate appearance of a band migrating in the MAP2a position, the new MAP2 species gradually was elevated from its previous electrophoretic position. Note in particular the indistinct nature of the MAP2 band at day 15 in both the cerebellum and the cerebral cortex (Fig. 3). The nature of this change from a sharply resolved single form through an indistinct intermediate to a sharply resolved doublet suggested a post-translational mechanism. Since adult MAP2 is known to be a phosphoprotein and since cAMP-dependent protein kinase is known to be associated

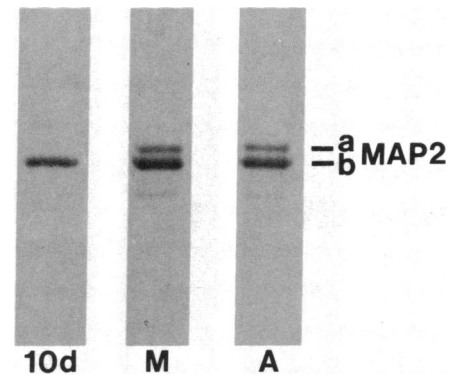


FIG. 5. Electrophoretic comigration of immature MAP2 with adult MAP2b. Taxol-stabilized microtubules from postnatal day 10 and adult rat brains were mixed and coelectrophoresed in the same slab gel slot prior to transfer to nitrocellulose. MAP2 was visualized by using AP-7. Microtubules from 10-day-old brains (lane 10d) exhibited a single immunoreactive species of MAP2, whereas those from adult brain (lane A) exhibited the MAP2a and MAP2b doublet. When mixed and electrophoresed (lane M), the MAP2b band was more heavily stained, indicating comigration of immature MAP2 with adult MAP2b.

with adult MAP2 (2, 21), phosphorylation was an obvious candidate for induction of the MAP2 doublet.

Although much work has been done on the *in vitro* phosphorylation of MAP2, no documentation existed suggesting that phosphorylation induces a change in its electrophoretic mobility. Therefore, to determine whether phosphorylation could cause such a change, taxol-stabilized adult rat brain microtubules were phosphorylated in the absence and presence of cAMP (12). Electrophoresis demonstrated little change in mobility upon cAMP-independent phosphorylation (Fig. 6, lane B); however, a shift to an apparent higher molecular weight of both adult MAP2 polypeptides was in evidence when phosphorylation was performed in the presence of cAMP (Fig. 6, lane C). This change in mobility was confirmed when samples from both cAMP-independent and cAMP-dependent phosphorylation were mixed after NaDodSO₄ treatment and electrophoresed in the same well (Fig. 6, lane D), resulting in a triple band. A similar experiment was performed on taxol-stabilized microtubules assembled from whole 10-day-old rat brains. Samples phosphorylated for 20, 40, and 60 min in the presence of ³²P-labeled ATP and cAMP

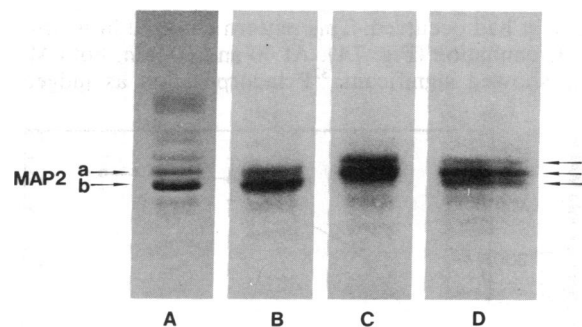


FIG. 6. Cyclic AMP-dependent phosphorylation causes adult MAP2 to migrate at an apparently higher molecular weight. Lanes: A, Coomassie blue stain of the high molecular weight region of an adult taxol-stabilized microtubule preparation; B, autoradiograph of a preparation identical to that in lane A but phosphorylated for 10 min in the absence of cAMP; C, autoradiograph of a preparation identical to that in lane A but phosphorylated for 10 min in the presence of cAMP; D, mixture of preparations in lanes B and C coelectrophoresed in the same well. All samples were run on the same slab gel.

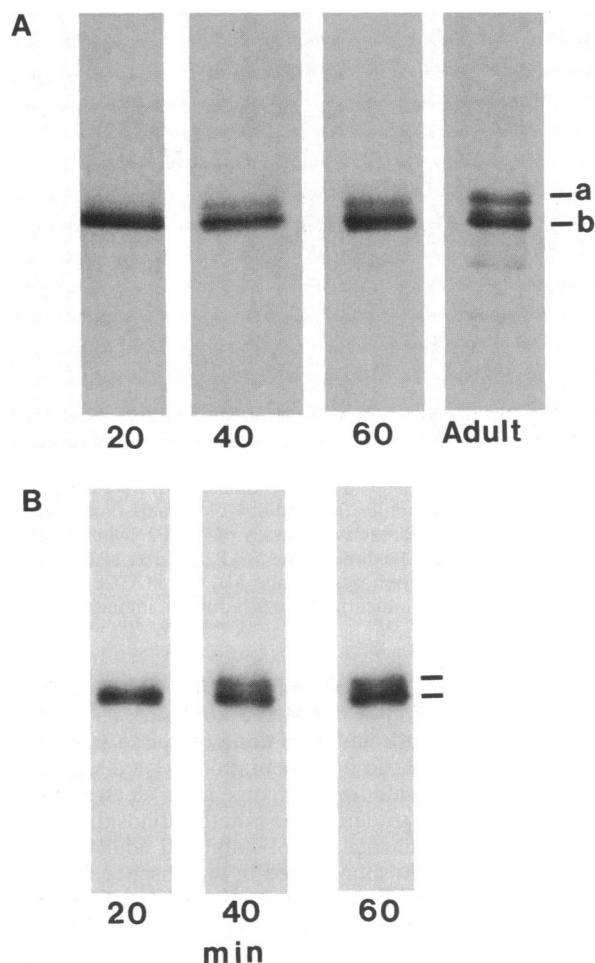


FIG. 7. Cyclic AMP-dependent phosphorylation causes immature MAP2 to resolve into two electrophoretic species. (A) Phosphorylation time course as seen by immunoblotting with a MAP2 monoclonal antibody. A nonphosphorylated adult control is shown at the far right. (B) Autoradiography of samples from the immunoblot shown in A. Note that both bands are labeled with ³²P.

were electrophoresed, transferred to nitrocellulose, stained with a MAP2 monoclonal antibody (Fig. 7A) and then autoradiographed (Fig. 7B). After 20 min of phosphorylation, little change in mobility was observed, but by 40 min a definitive band split had occurred. This pattern changed little after 60 min of incubation (Fig. 7A). At 40 and 60 min, both MAP2 bands showed significant ³²P incorporation as judged by

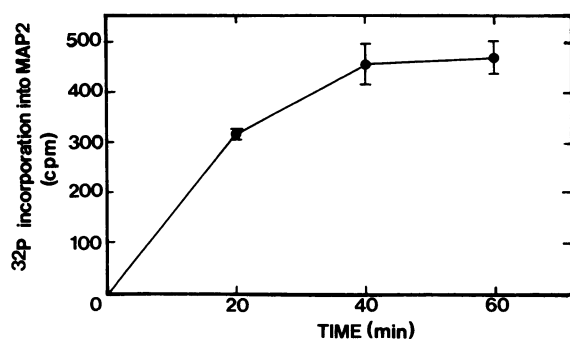


FIG. 8. Time course of the MAP2 phosphorylation depicted in Fig. 7. The MAP2 band or bands identified by blotting were excised and counted in a scintillation counter. Triplicate samples were counted at each time point, and the data were expressed as mean cpm \pm SD.

autoradiography (Fig. 7B). Incorporation of phosphate into MAP2 had reached plateau after 40 min of incubation (Fig. 8). Since little additional phosphate was incorporated after MAP2 had split into two bands, it was apparent that phosphorylation resolved two distinct species of MAP2.

DISCUSSION

MAP2 changes its electrophoretic mobility during development of the rat brain. This alteration is not simultaneous throughout the nervous system because the spinal cord contains MAP2 in the adult configuration by day 10, while the rest of the brain undergoes change to the adult form of MAP2 between days 17 and 25. A splitting of the MAP2 band, which is nearly identical to that observed in the adult species of MAP2 *in vivo*, can be induced by *in vitro* phosphorylation of 10-day MAP2, indicating that timed phosphorylation events may be responsible for the observed change in MAP2 during development.

MAP2 isolated from adult brain contains 8–10 mol of phosphate per 300,000 g of protein. Additionally, 10–13 phosphates can be incorporated into this protein upon phosphorylation in the presence of cAMP and a kinase that is intimately associated with MAP2 (12, 24, 25). We demonstrate that cAMP-induced phosphorylation *in vitro* shifts adult MAP2 to an apparent higher molecular weight. That a shift in electrophoretic mobility of MAP2 after phosphorylation has not been previously reported is undoubtedly due to the use of high percentage NaDodSO₄/polyacrylamide gel systems by most investigators. Such gels resolve the high molecular weight region poorly and will not exhibit relatively small increases in apparent molecular weight due to phosphorylation.

The change of MAP2 from a single band, comigrating with adult MAP2b, to a double band *in vivo* does not occur precipitously but rather is gradual. At postnatal day 15, an indistinct region of MAP2 immunoreactivity replaces the sharply resolved immature MAP2 species apparent at postnatal days 10 and 13 (see Figs. 1 and 3). By postnatal day 25, MAP2 has separated further and appears in its adult configuration (Fig. 3). *In vitro* phosphorylation of immature MAP2 did induce the formation of a double band. However, it should be noted that the separation of putative MAP2a and 2b is not as extensive as that observed in either NaDodSO₄ extracts from adult brain regions (Figs. 1 and 3) or adult taxol-stabilized microtubules (Fig. 7A). Rather it is similar to that seen in NaDodSO₄ extracts of 17–18 day cerebella and cerebral cortices (Fig. 3). Although these two bands may represent unique primary transcripts of MAP2 genes, the possibility exists that they are actually different phosphorylation states of the same polypeptide. This appears unlikely because the level of phosphate incorporation (Fig. 8) had reached a plateau after the resolution of the two bands occurred (Fig. 7), indicating that two unique species of MAP2 were differentiated from each other by phosphorylation. Finally, if the electrophoretic alteration of immature MAP2 *in vivo* (Fig. 3) is indeed due to timed phosphorylation events, our *in vitro* phosphorylation experiments (Figs. 7 and 8) suggest that an alteration in kinase activity or the expression of a different kinase at or near postnatal day 17 may be necessary to achieve the adult configuration of MAP2.

It is noteworthy that the phosphorylation-induced band splitting of immature MAP2 (Fig. 7) occurs after *ca.* 70% of the equilibrium amount of phosphate has been incorporated (Fig. 8). This suggests that the *in vitro* resolution of the two electrophoretic species is dependent upon either the attainment of a critical number of phosphates or the phosphorylation of specific serine and/or threonine residues. Others have shown that phosphatase treatment of adult MAP2 does not result in the removal of all endogenous phosphate groups

(10), suggesting that there are different classes of phospho amino acids on these polypeptides.

Should phosphorylation prove to be the mechanism by which MAP2 is altered from a single to a double band during development, certain functional changes based on *in vitro* studies of MAP2 are suggested. Seldon and Pollard (10) have shown that the ability of MAP2 to induce actin gelation is dependent upon its phosphorylation state. These studies indicate that dephosphorylated MAP2 stimulates actin gelation more efficiently than does MAP2 with its adult complement of phosphates. If the alteration from a single to a double band of MAP2 *in vivo* is caused by phosphorylation, it follows that immature MAP2 is at a lower phosphorylation state than is adult MAP2. This suggests that immature MAP2, when compared with adult MAP2, may interact more readily with actin *in vivo*. The affinity of MAP2 for microtubules is also affected by phosphorylation, in that highly phosphorylated MAP2 more readily elutes from taxol-stabilized microtubules than does its less phosphorylated form (11). Since MAP2 appears to stabilize the microtubule polymer (26, 27), its phosphorylation state may provide a mechanism that governs the stability of microtubules *in vivo*.

Other changes occurring in the microtubule proteins during development are also described briefly in this report. For example, MAP1, the largest HMW MAP, is largely absent from taxol-stabilized microtubules made from the brains of 10-day old rat pups, whereas, a novel MAP, MAPX (Fig. 4), is present in significant quantities. A major importance of our studies is that they support a growing body of evidence which shows that much of the microtubule system (i.e., tubulin, MAP2, and τ) is dramatically altered during brain maturation (17, 28–31). In order to understand microtubule functions, the relationship between tubulin and its regulatory proteins requires analysis. The developing brain is an ideal model system in which to study this relationship because different tubulins and different forms of MAPs can be biochemically isolated and analyzed at different stages of development.

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