Characterization of Microtubule-Associated Protein MAP1B: Phosphorylation State, Light Chains, and Binding to Microtubules†

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ABSTRACT: We have recently described a procedure for the purification of microtubule associated protein 1B (MAP1B) from calf brain [Pedrotti, B., & Islam, K. (1995) *Cell Motil. Cytoskeleton 30*, 301−309], and this study further characterizes the purified protein and its interaction with microtubules. We show that purified MAP1B (1) is thermostable; (2) is mainly phosphorylated at the casein kinase II (CKII) sites but only partially phosphorylated at the proline-directed protein kinase (PDPK) sites; (3) both the CKII and PDPK sites can be dephosphorylated by alkaline phosphatase; and (4) dephosphorylation results in an increased mobility on SDS−PAGE gels. The ability of MAP1B to interact with microtubules was also examined and shows that (1) phosphorylated (1B-P), alkaline phosphatase-treated (1B-AP), and heat-treated (1B-P), alkaline phosphatase-treated (1B-AP), and heat-treated (1B-HT) MAP1B bind to taxol-stabilized microtubules; (2) 1 mol of 1B-P, 1B-AP, or 1B-HT each binds about 13−14 tubulin dimers; (3) light chain interaction with MAP1B heavy chain is not affected by AP- or heat-treatment; (4) MAP1B can be displaced from taxol-stabilized microtubules by titration with salt; (5) higher salt concentrations are required to displace 1B-AP compared with 1B-P from taxol-stabilized microtubules; and (6) MAP2 is able to displace both 1B-P and 1B-AP from taxol-stabilized microtubules. The role of phosphorylation in regulating MAP1B interaction with microtubules and light chains is discussed.

A heterogeneous group of proteins (MAPs) normally associated with microtubules have been suggested to play a critical role in microtubule assembly and in the maintenance of neuronal processes [for reviews see Wiche (1989) and Tucker (1990)].[1] Microtubule-associated protein 1B [MAP1B, also known as MAP1X, MAP1.2, MAP5; see Muller et al. (1994) for references and nomenclatures] differs from most high molecular weight MAPs in that it is mainly present in neurons at early developmental stages and in the relative inefficiency with which it co-sediments with microtubules in vitro. However, antisera against MAP1B immunostain microtubule networks in neuronal cells, axons and dendrites, and non-neuronal cell lines (Wiche, 1989).

MAP1B is the earliest MAP to appear during brain development and is the most abundant in fetal and neonatal brains (Matus, 1988; Schoenfeld et al., 1989) but is found at only low basal levels in adult brain. However, MAP1B persists in adult brain areas with unusually high regenerative activities (Viereck et al., 1989). The strong developmental regulation of MAP1B and its prominence in newly forming neuritic processes suggest a role in neurogenesis and process plasticity. This suggestion is further supported by experiments with PC12 cell line in which the induction of neurite outgrowth by nerve growth factor (NGF) results in a rise in the level of MAP1B (Aletta et al., 1988; Brugg & Matus, 1988). More recently, Brugg et al. (1993) have shown that attenuation of MAP1B expression by antisense oligodeoxynucleotides inhibits initiation of neurite outgrowth. MAP1B expression has also been demonstrated to precede the development of the mature oligodendrocyte phenotype (Vouyiokalis & Brophy, 1993), suggesting a role in the formation and stabilization of myelin-forming processes. In addition, alterations in the expression of MAP1B have been implicated in schizophrenia (Arnold et al., 1991), and hyperphosphorylated MAP1B has been found associated with neurofibrillary tangles in Alzheimer's disease (Hagesawa et al., 1990; Ulloa et al., 1994a).

The primary sequence of MAP1B indicates the presence of multiple sites which may undergo phosphorylation (Noble et al., 1989). Indeed, phosphopeptide patterns of MAP1B show the presence of numerous phosphate residues (Diaz-Nido et al., 1988), although the precise sites of phosphorylation are not known. On the basis of the electrophoretic behavior of MAP1B two major modes of MAP1B phosphorylation have been identified, termed mode I, which results in a decreased migration of MAP1B on SDS−PAGE, and mode II, which hardly modifies the electrophoretic behavior of the protein (Ulloa et al., 1994b). Several proline-directed protein kinases (PDPK), e.g., NGF-dependent high molecular

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MAP(s), microtubule-associated protein(s); MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Tris, Trizma base; PMSF, phenylmethanesulfonyl fluoride; CKII, casein kinase II; PDPK, proline-directed protein kinase; AP, alkaline phosphatase.

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weight MAP kinase (HMK; Tsao et al., 1990) and MAP1B kinase (M1BK; Hoshi et al., 1990), have been shown to phosphorylate MAP1B at mode I sites, while mode II sites have been shown to be phosphorylated by casein kinase II (Diaz-Nido et al., 1988; Ulloa et al., 1994b). Phosphorylated MAP1B has been reported to exhibit an increased association with assembled microtubules in vivo (Brugg & Matus, 1988) and in vitro (Diaz-Nido et al., 1988; Ulloa et al., 1993b), and it has been suggested that phosphorylation by casein kinase II may favor MAP1B:microtubule interaction (Diaz-Nido et al., 1988; Ulloa et al., 1993b).

Monoclonal and polyclonal antibodies against phosphate-sensitive and phosphate-insensitive epitopes have been used to monitor the distribution of phosphorylated isoforms of MAP1B during brain development [see Ulloa et al. (1993a,b), Black et al. (1994), Gordon-Weeks et al. (1993), and Keating and Asai (1994)]. Mode I-phosphorylated MAP1B has been shown to be present only at initial stages of neuronal differentiation, related to axonal sprouting, and then becomes restricted to the distal region in developing axonal processes until it finally disappears when axon growth resumes. Stimulation by NGF leads to an increase in the amount of MAP1B and exhibits a concomitant increase in the amount of phosphorylated MAP1B (Greene et al., 1983). In view of its spatial and temporal distribution it has been suggested that mode I-phosphorylated MAP1B could be used as a marker for active axonal growth (Ulloa et al., 1994b). On the other hand, mode II-phosphorylated MAP1B is present not only in axons but also in dendrites and remains in late stages of neuronal maturation (Ulloa et al., 1994b; Riederer et al., 1993; Riederer, 1995). Antisense oligonucleotides for CKII prevent neurite extensions and lead to a diminished association of MAP1B with microtubules (Ulloa et al., 1993b). Mode II phosphorylation may therefore perform other more general functions such as microtubule stabilization, essential for neurite extension (Ulloa et al., 1993b). In addition to the kinases, MAP1B phosphorylation may also be regulated by protein phosphatases (PP), and in this context it is important to mention that in vitro mode I sites are efficiently dephosphorylated by PP2B but not by PP1, while mode II sites are dephosphorylated by PP1 but not by PP2B (Ulloa et al., 1993c).

The deduced protein sequence of MAP1B (Noble et al., 1989) shows a significant sequence homology with another high molecular weight protein, MAP1A (Langkopf et al., 1992), but not with MAP2 (Lewis et al., 1988). Positively charged KKE repeat motifs in the N-terminal portion of MAP1B, similar to those in MAP1A, have been suggested to constitute the microtubule binding site (Langkopf et al., 1992; Noble et al., 1989), although additional microtubule binding sites have recently been observed for MAP1A (Cravchik et al., 1994). Both proteins have a thin filamentous shape (Shiomura & Hirokawa, 1987; Sato-Yoshitake et al., 1989) and contain two common associated light chains, LC1 and LC3, of 32 and 18 kDa, respectively (Schoenfeld et al., 1989; Pedrotti & Islam, 1994, 1995). MAP1B/LC1, like MAP1A/LC2, is encoded in a single mRNA, and the polyprotein precursor is probably post-translationally processed to produce the mature proteins (Langkopf et al., 1992; Hammarback et al., 1991). LC3 is encoded on a separate mRNA (Mann & Hammarback, 1994).

The lack of satisfactory purification procedures has limited any detailed biochemical studies with MAP1B. Our laboratory has recently described a procedure for the purification of MAP1B and demonstrated that the purified MAP1B can bind to microtubules (Pedrotti & Islam, 1995). As MAP1B is known to be phosphorylated by different protein kinases and phosphorylation may modulate the interaction of MAP1B with microtubules, we have sought to characterize the phosphorylation state of the purified MAP1B protein using several phosphate-sensitive monoclonal and polyclonal antibodies. Our results indicate that the purified protein is phosphorylated, and we have therefore further examined the affect of phosphorylation on light chain composition and interaction with microtubules by comparing native phosphorylated MAP1B protein with MAP1B dephosphorylated by alkaline phosphatase treatment. As dephosphorylation of MAP1B requires a thermal treatment step the thermostability of the purified MAP1B has also been examined.

MATERIALS AND METHODS

The following buffers were used: MES buffer (0.1 M MES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT, pH 6.4, with NaOH) and PIPES buffer (0.1 M PIPES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT, pH 6.9, with NaOH). All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and all chemicals used were of Analar grade.

Protein Purification. Calf-brain microtubule protein was prepared by two cycles of temperature dependent assembly/disassembly according to Islam and Burns (1981) as modified by Pedrotti et al. (1993). Briefly, whole brain (about 200 g wet weight) was homogenized in PIPES buffer, containing 0.1 mM PMSF and 40 μg of leupeptin/mL. After centrifugation the supernatant was adjusted to 1 mM GTP and incubated at 37 °C for 20 min. At the end of the incubation period the assembled protein was collected by centrifugation and the supernatant was decanted and used for purification of MAP1B (see below). The pellets containing the assembled microtubules were resuspended in MES buffer and cold-dissociated at 4 °C for 60 min. After removal of the cold-stable material by centrifugation, the supernatant was adjusted to 1 mM GTP, in the absence of glycerol, and all other steps were performed in MES buffer as described previously (Islam & Burns, 1981). Microtubule protein (15−20 mg/mL) was frozen and stored in liquid nitrogen.

Tubulin, MAP2, and MAP1A were purified as described previously (Pedrotti et al., 1994a; Pedrotti & Islam, 1994).

MAP1B was purified as described in Pedrotti and Islam (1995). Briefly, the supernatant after the first warm centrifugation step (see above) was further centrifuged for 12 h at 80 000*g* at 4 °C and loaded onto a Mono-Q column (Pharmacia 10/10). The unbound protein was removed by washing the column with MES buffer containing 0.2 M NaCl, and bound protein was eluted with salt-steps of 0.3 and 0.4 M NaCl. The 0.4 M salt-eluted protein was dialyzed to remove the salt and subsequently loaded onto a Mono-S column. The MAP1B protein (>95% pure) was eluted at 0.33 M NaCl, dialyzed against MES buffer, and stored in liquid nitrogen.

Thermal treatment of brain supernates and purified MAP1B was performed according to the procedure of Herzog and Weber (1978), and alkaline phosphatase treatment was carried out according to the procedure of Ulloa et al. (1993a).

Binding Studies. Taxol-stabilized microtubules were prepared using purified tubulin as previously described

(Pedrotti & Islam, 1994). Taxol-stabilized microtubules were sedimented by centrifugation, analyzed by SDS−PAGE, and densitometrically scanned as described in Pedrotti and Islam (1994, 1995). Solid-phase immunoassays, using the MAP1B-4 monoclonal antibody, were performed as previously described (Pedrotti et al., 1994a; Pedrotti & Islam, 1995).

Antibodies. MAP1B. Monoclonal antibodies against phosphoepitopes present in the MAP1B molecule and polyclonal antibodies raised against phosphorylatable regions in MAP1B, in unmodified form, have been described previously (Ulloa et al., 1994b). Monoclonal antibody MAP1B-4 against phosphorylation-independent epitope was purchased from Amersham (Arlington Heights, IL).

Monoclonal HM-1 and τ-2 antibodies were purchased from Sigma Chemical Co. (St. Louis, MO), while anti-MAP1A, anti-MAP2.3, and horseradish peroxidase-labeled anti-mouse IgG were purchased from Amersham.

Protein Composition and Western Blotting. Proteins were fractionated by denaturing SDS−PAGE using the Pharmacia Phast system. The gels were stained, and the integrated peak areas were determined as previously described (Pedrotti & Islam, 1994). The fractionated proteins were also transferred onto nitrocellulose membranes and immunostained using monoclonal antibodies against MAP2, MAP1A, MAP1B, τ (Pedrotti & Islam, 1994), and monoclonal and polyclonal antibodies against MAP1B phosphorylation epitopes (Ulloa et al., 1993a). Alkaline phosphatase treatment was as described by Ulloa et al. (1993a).

Protein concentration was determined using the Bradford method (Bio-Rad protein reagent kit), and bovine serum albumin was used as the standard.

RESULTS

Characterization of MAP1B Protein. Protein Composition. MAP1B in our preparations typically consists of a high molecular mass (apparent M_r of 330 kDa) heavy chain and two low molecular mass (apparent M_r of 32 and 18 kDa, termed LC1 and LC3, respectively) light chains. The relative stoichiometry of 2 mol of LC1/mol of heavy chain was fairly constant in different preparations of MAP1B while that of LC3 varied between 0.2 and 0.5 mol of LC3/mol of heavy chain [see below and Pedrotti and Islam (1995)]. Immunostaining of purified MAP1B, by dot-blotting onto nitrocellulose filters, showed that the protein was recognized only by the MAP1B-4 antibody and not by the τ-2 or MAP2.3 monoclonal antibodies (not shown). Western blotting using the HM-1 (anti-MAP1A) and AA6 (anti-MAP1B) antibodies showed that only the AA6 antibody stained the purified protein (see below). MAP1B used in this study therefore does not contain detectable amounts of MAP1A, MAP2, or τ proteins [see also Pedrotti and Islam, (1995)].

Thermal Stability. Bulk MAP1 proteins have been reported to be either heat-labile (Kuznetsov et al., 1981) or heat-stable (Vera et al., 1988), although Vallee (1985) has suggested that it depends on the presence of other proteins. Indeed, we have recently shown that MAP1A is heat-labile in crude brain supernates but heat-stable once purified (Pedrotti & Islam, 1994). We therefore examined the heat-lability of MAP1B using crude brain supernates and purified MAP1B. SDS−PAGE analysis of untreated and heat-treated crude brain supernates showed that while both MAP1 and MAP2 were present in the untreated supernate only MAP2 was present in the heat-treated supernate. The absence of MAP1 protein in the heat-treated supernate was further confirmed by the observation that MAP1B was immunostained only in the untreated supernate and not in the heat-treated supernate (Figure 1). By contrast, SDS−PAGE analysis of heat-treated purified MAP1B showed that the protein remained in the supernatant (Figure 1), little or no MAP1B was found to pellet (data not shown), suggesting that purified MAP1B is intrinsically heat-stable.

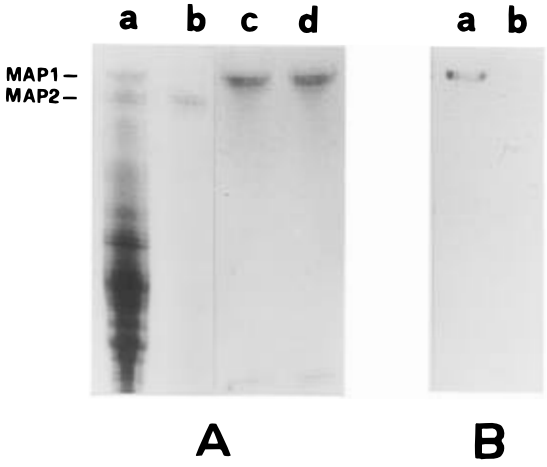

FIGURE 1: Effect of heat-treatment on MAP1B in crude brain supernates and purified MAP1B. Heat-treatment of whole brain supernates and purified MAP1B, 100 °C for 5 min, was carried out essentially as described by Vera et al. (1988). The protein was fractionated on 4%−15% acrylamide gradient gels. (A) Coomassie-stained brain supernate before (lane a) and after (lane b) heat-treatment and purified MAP1B before (lane c) and after (lane d) heat-treatment. (B) Brain supernate before (lane a) and after (lane b) heat-treatment was also transferred onto a nitrocellulose membrane and immunostained with the AA6 (anti-MAP1B) monoclonal antibody.

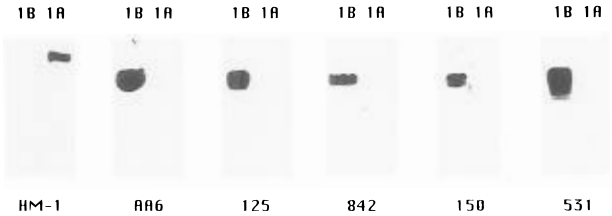

FIGURE 2: Immunostaining of purified MAP1A and MAP1B by different monoclonal and polyclonal antibodies. Purified MAP1A or MAP1B was fractionated by SDS−PAGE on a 7.5% acrylamide gel, under denaturing conditions, and blotted onto nitrocellulose membranes. The membranes were immunostained using the HM-1 (anti-MAP1A), AA6 (anti-MAP1B), 125, 842, 150, and 531 antibodies.

Phosphorylation State. The phosphorylation state of purified MAP1B was examined using several phosphate-sensitive and -insensitive monoclonal and polyclonal antibodies [see Ulloa et al. (1993a)]. In order to determine the specificity of these antibodies purified MAP1A and MAP1B were blotted onto nitrocellulose membranes and probed using the 125, 842, 150, and 531 antibodies as well as the anti-MAP1A (HM-1) and anti-MAP1B (AA6) antibodies. As shown in Figure 2, HM-1 antibody immunostained only MAP1A and not MAP1B, thereby confirming that MAP1B does not contain detectable amounts of MAP1A. Furthermore, AA6, 125, 842, 150, and 531 antibodies exclusively stained MAP1B and did not cross-react with MAP1A.

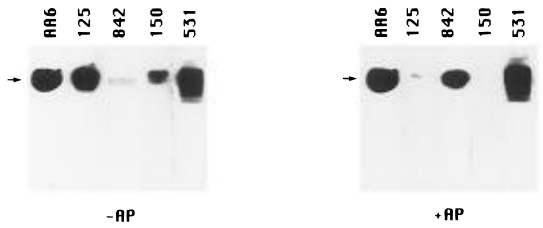

FIGURE 3: Immunostaining of MAP1B before (−AP) and after (+AP) alkaline phosphatase treatment of nitrocellulose membranes by the indicated monoclonal and polyclonal antibodies.

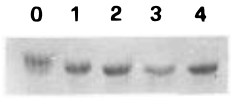

FIGURE 4: SDS−PAGE analysis of MAP1B after different times of AP-treatment. Purified MAP1B was incubated either in the absence (0) or in the presence of 0.26 units of AP/μg of protein for up to 4 h (1−4). Aliquots were removed at 1 h intervals, and the reaction was stopped by addition of Laemmli buffer and heating at 100 °C for 5 min. The protein was fractionated by SDS−PAGE on a 7.5% acrylamide gel and stained with Coomassie Brilliant Blue.

The various monoclonal anbitodies recognize different phosphorylation states of MAP1B: AA6 is phosphate-insensitive, 125 is a CKII−phosphorylated epitope(s), and 150 is a phosphorylated PDPK epitope(s). Similarly, the polyclonal antibodies 531 and 842 recognize an unphosphorylated PDPK and CKII epitope(s), respectively, but fail to recognize the phosphorylated epitope(s). As shown in Figure 3 antibodies AA6, 531, and 125 intensely stained MAP1B while antibodies 842 and 150 show a substantially lower staining. These differences in staining are not due to differences in the affinity of the various antibodies; for example, 842 antibody has a higher affinity compared with 150 and 531 and a lower affinity when compared with 125 (Ulloa et al., 1993a). Taken together, these data would suggest that the CKII sites are largely phosphorylated while PDPK sites are only partially phosphorylated.

To further confirm that this was the case, the membranes were treated with alkaline phosphatase (AP) and subsequently restained using the same antibodies. No difference in staining by the AA6 antibody was observed before or after AP-treatment (Figure 3), indicating that AP-treatment does not affect antibody binding. By contrast, AP-treatment totally abolished staining by antibodies 125 and 150 (Figure 3), thereby confirming that the protein was dephosphorylated. Moreover, the staining by antibody 531 was only minimally enhanced after AP-treatment but significantly enhanced for antibody 842 (Figure 3).

Time Course of Dephosphorylation. In view of the observations that MAP1B was phosphorylated, heat-stable, and could be dephosphorylated by alkaline phosphatase we also performed a time course of dephosphorylation of the protein. Purified MAP1B was therefore incubated either in the absence of alkaline phosphatase or in its presence for 4 h. Aliquots were removed at the indicated times, and the reaction was stopped by addition of sample buffer and boiling for 5 min at 100 °C. SDS−PAGE analysis (Figure 4) showed an increase in mobility of the MAP1B protein with increasing time of alkaline phosphatase treatment. The MAP1B protein band, which prior to dephosphorylation appeared diffuse, tended to become sharper after dephosphorylation.

The protein was also blotted onto nitrocellulose membranes and stained using the monoclonal and polyclonal antibodies in order to determine the time course of dephosphorylation of the different phosphorylated epitopes. As shown in Figure 5, antibody 150 (Figure 5D) failed to immunostain MAP1B after 1 h of AP-treatment. The epitope(s) for antibody 125 also decreased with increasing times of dephosphorylation, the reaction disappeared in MAP1B incubated for 3 h with AP, while that for antibody 842 progressively increased (cf. Figure 5B,C). By contrast, epitopes for AA6 (Figure 5A) and 531 (Figure 5E) antibodies showed relatively little change. These observations suggest that the epitope(s) for 150 antibody was dephosphorylated rapidly while that for antibodies 125 and 842 exhibited slower kinetics of dephosphorylation. In general, it would appear that dephosphorylation of the epitope(s) recognized in the unmodified form by 842 antibody was slower than that for the epitope(s) for 125 antibody. Indeed, the maximal intensity of signal with 842 antibody was achieved at longer time periods when compared with the loss of staining with 125 antibody. The behavior of the different antibodies further confirms that after 3−4 h of AP-treatment MAP1B protein was essentially dephosphorylated, even though alkaline resistant phosphates may still be present.

Interaction of MAP1B with Microtubules. Binding of Phosphorylated (1B-P), Dephosphorylated (1B-AP), and Heat-Treated MAP1B (1B-HT) to Taxol-Stabilized Microtubules. The effect of different treatments on the interaction with taxol-stabilized microtubules was also examined by sedimentation. Pure tubulin was polymerized in the presence of 20 μM taxol and 500 μM GTP for 20 min and then incubated for a further 10 min in the presence of 1B-P, 1B-AP, or 1B-HT. At the end of the incubation period, taxol-stabilized microtubules were sedimented by centrifugation

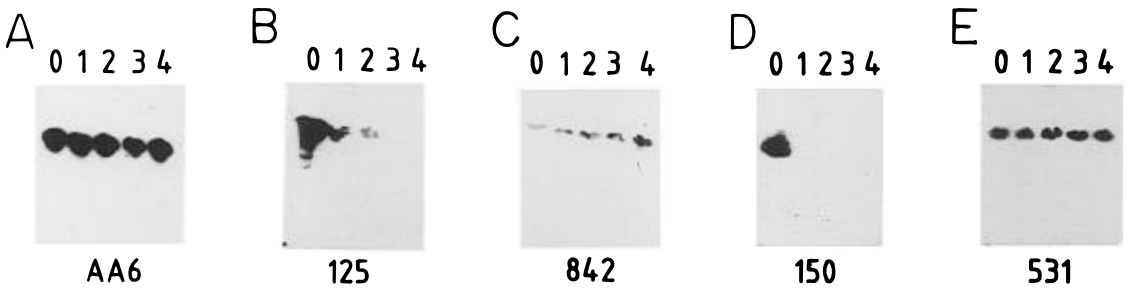

FIGURE 5: Immunoblotting of MAP1B with the indicated antibodies after various times of AP-treatment. Purified MAP1B was incubated either in the absence (0) or in the presence (1−4) of 0.26 units of AP/μg of protein. Aliquots were removed at 1 h intervals (0−4 h) and heated at 100 °C for 5 min in Laemmli buffer. The protein was fractionated by SDS−PAGE on 7.5% acrylamide gels, transferred onto nitrocellulose membranes, and immunostained using the AA6 (A), 125 (B), 842 (C), 150 (D), and 531 (E) antibodies.

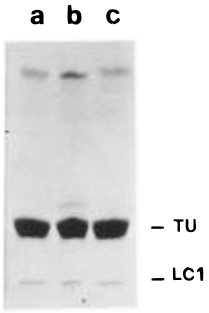

FIGURE 6: Binding of 1B-P, 1B-AP, and 1B-HT to taxol-stabilized microtubules. Taxol-stabilized microtubules were incubated with 1B-P, 1B-AP, or 1B-HT and pelleted by centrifugation. The pellets were fractionated by SDS−PAGE on 4%−15% acrylamide gradient gels and stained with Coomassie Blue: (a) 1B-P, (b) 1B-AP, and (c) 1B-HT. The positions of tubulin (Tu) and LC1 are indicated.

and the pellets and supernatants were examined by SDS-PAGE. As shown in Figure 6, MAP1B co-sedimented with microtubules irrespective of treatment, suggesting that both phosphorylated and dephosphorylated 1B can bind to microtubules and that heat-treatment does not affect the ability of the protein to interact with microtubules. When 1B-P, 1B-AP, and 1B-HT were incubated and sedimented under identical conditions but in the absence of microtubules, MAP1B protein was only observed in the supernatants and did not pellet (data not shown).

The stoichiometry of MAP1B to tubulin dimers in the pelleted microtubules was estimated by densitometrically scanning the Coomassie-stained gels and determination of the integrated peak areas. The molecular abundance was calculated after correction for the molecular mass of MAP1B (244 kDa) and tubulin (100 kDa). One mole of 1B-P, 1B-AP, and 1B-HT bound 14.2 (±0.72), 13.5 (±0.66), and 14.3 (±0.86) mol of tubulin dimers respectively, suggesting that neither heat-treatment nor dephosphorylation significantly altered the ability of the protein to interact with microtubules.

Light Chains. MAP1B has been shown to contain two associated light chains termed LC1 and LC3 (Schoenfeld et al., 1989; Pedrotti & Islam, 1995). The affect of AP- or heat-treatment on the composition of the light chains was also examined. 1B-P, 1B-AP, and 1B-HT were fractionated by SDS−PAGE on 20% acrylamide gels and, after staining and destaining, were densitometrically scanned. The integrated peak areas were determined and used to calculate the ratio of LC3 and LC1 (see Figure 7 and Table 1). There appears to be little or no difference in the amount of light chains or in their respective ratio for 1B-P, 1B-AP, or 1B-HT. The relative amounts of LC1 and LC3 in 1B-P, 1B-AP, and 1B-HT that co-sedimented with taxol-stabilized microtubules were also determined. As shown in Table 1 the ratio of LC1:LC3 was roughly 1:0.25 in all cases. These data suggest that the phosphorylation state of MAP1B does not influence the association between the heavy chain and light chains.

Effect of NaCl on MAP1B Binding to Taxol-Stabilized Microtubules. MAPs can be removed from taxol-stabilized microtubules by NaCl [see Pedrotti et al. (1994a)]. Moreover, salt-titration experiments have shown that phosphorylated MAP2 binds less strongly to microtubules compared with non-phosphorylated MAP2 (Burns et al., 1984). We therefore examined the ability of NaCl to displace microtubule-bound MAP1B after different times of AP-treatment.

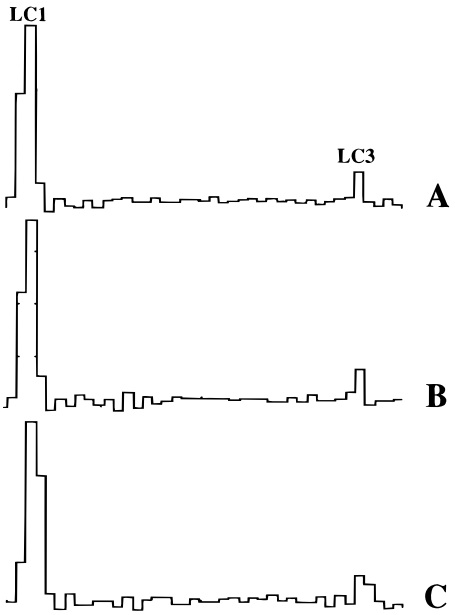

FIGURE 7: Light chain composition of 1B-P, 1B-AP, and 1B-HT. 1B-P (A), 1B-AP (B), and 1B-HT (C) were fractionated by SDS−PAGE on 20% acrylamide gels, stained with Coomassie Blue, and densitometrically scanned. The positions of LC1 and LC3 are indicated.

Table 1: Light Chain Composition of Initial and Microtubule-Associated 1B-P, 1B-AP, and 1B-HT[a]

protein sample	heavy chain	LC1	LC3
1B-P	1.0	2.20 ± 0.25	0.57 ± 0.047
1B-AP	1.0	2.06 ± 0.16	0.51 ± 0.024
1B-HT	1.0	2.03 ± 0.14	0.47 ± 0.071
1B-P + Mts	1.0	2.02 ± 0.33	0.39 ± 0.011
1B-AP + Mts	1.0	1.92 ± 0.10	0.37 ± 0.018
1B-HT + Mts	1.0	2.07 ± 0.22	0.38 ± 0.004

[a] The composition of either initial 1B-P, 1B-AP, and 1B-HT or that co-sedimenting with taxol-stabilized microtubules was analyzed after fractionation by SDS−PAGE on 4%−15% acrylamide gradient gels. The gels were stained with Coomassie Brilliant Blue and densitometrically scanned. The molar ratio of LC1 and LC3 relative to the heavy chain was calculated from the integrated peak areas.

MAP1B was dephosphorylated for the indicated times and incubated with taxol-stabilized microtubules. MAP1B treated under identical conditions but in the absence of AP was used as a control. At the end of the incubation period the microtubules were diluted 1:1 in MES buffer containing 400 mM NaCl. The taxol-stabilized microtubules were pelleted and after resuspension the pelleted protein was analysed by SDS−PAGE. The gels were densitometrically scanned to determine the amount of bound MAP1B. As shown in Figure 8, the amount of MAP1B which co-sediments with taxol-stabilized microtubules in the presence of 400 mM NaCl increased 1.6-fold after the first hour of AP-treatment when compared with the control. Only a slight decrease in MAP1B binding was observed when the protein was first incubated for 3 h with AP, the protein was no longer recognized by antibody 125.

In a separate experiment, the effect of NaCl on the binding of phosphorylated (1B-P) and dephosphorylated MAP1B (1B-AP) was also examined using the solid-phase immunoassays (SPI). Microtiter wells coated with 0.1 mg of taxol-stabilized microtubules/mL or buffer were, after blocking with 5% glycine, challenged with 30 μg of 1B-P or 1B-AP/

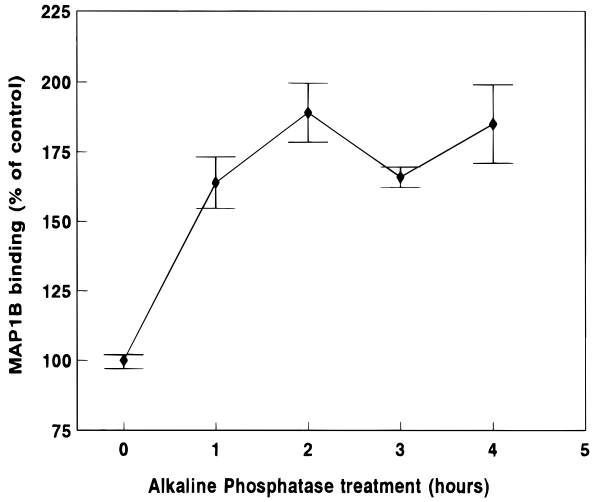

FIGURE 8: Binding of 1B-P and 1B-AP to taxol-stabilized microtubules in the presence of NaCl. MAP1B was incubated either in the presence or absence of alkaline phosphatase (control) for the indicated times and then heated for 15 min at 65 °C. A 9 μg amount of either control or AP-treated MAP1B was incubated with 60 μg of taxol-stabilized microtubules in the presence of 200 mM NaCl. After centrifugation (see Materials and Methods) the pellets were fractionated by SDS−PAGE, stained with Coomassie Blue, and densitometrically scanned. The integrated peak areas were determined and the amount of microtubule-bound MAP1B expressed as a percentage of the control MAP1B. The values are a mean of 5−6 determinations.

mL, either in MES buffer or MES buffer containing the indicated amount of NaCl. The amount of microtubule-bound MAP1B was determined using the monoclonal MAP1B-4 antibody. Wells that had not been coated with microtubules gave a low background noise (absorbance at 492 nm < 0.2), while microtubule coated wells gave a signal for specific binding. In the absence of NaCl the specific binding expressed as the signal/background ratio was similar for 1B-P and 1B-AP (Figure 9a), although in the presence of NaCl the amount of microtubule-bound MAP1B decreased as a function of the salt concentration (Figure 9b). The amount of NaCl required to displace 50% of 1B-P (140 mM) was lower when compared with that for 1B-AP (185 mM), suggesting that 1B-AP binds much more strongly to microtubules compared with 1B-P.

Competition between MAP1B and MAP2 for Microtubule Binding. We have previously shown that co-incubation of MAP2 and MAP1B leads to a substantial decrease in the amount of microtubule-bound MAP1B (Pedrotti & Islam, 1995), suggesting that the two proteins may compete for a similar or overlapping site on the tubulin molecule [see also Cross et al. (1991)]. In view of the higher affinity of 1B-AP for microtubules we undertook experiments to determine whether MAP2 was able to displace both 1B-P and 1B-AP from microtubules. Taxol-stabilized microtubules were therefore incubated with 1B-AP or 1B-P, either in the presence or in absence of MAP2. At the end of the incubation time, taxol-stabilized microtubules were sedimented through a 30% sucrose cushion, and the pellets were analyzed by SDS−PAGE. As shown in Figure 10, the amount of both 1B-AP and 1B-P which co-sedimented with microtubules was considerably reduced in the presence of MAP2, suggesting that despite the higher affinity of 1B-AP to microtubules it was efficiently displaced by MAP2.

DISCUSSION

Despite indications that MAP1B may play an important role in the induction of neurite outgrowth, studies on its interaction with microtubules have been hampered by the lack of suitable purification protocols. We have recently described a procedure for the isolation of bovine brain MAP1B in milligram quantities and have shown that the purified protein can bind to microtubules (Pedrotti & Islam, 1995). As the phosphorylation state of MAP1B may modulate the binding to microtubules we have now further characterized the phosphorylation state of the MAP1B protein.

The observation that antibody 125, which recognizes the epitope(s) when it is phosphorylated, strongly stains the protein before but not after AP treatment argues that the epitope(s) was phosphorylated. Similarly, the fact that antibody 842, which recognizes only the dephosphorylated epitope(s), stains weakly before but strongly after AP-treatment indicates that this epitope(s) was also largely phosphorylated. As both antibodies recognize the CKII epitopes, it is probable that several CKII sites are phosphorylated. In contrast to the CKII sites, only one of the two

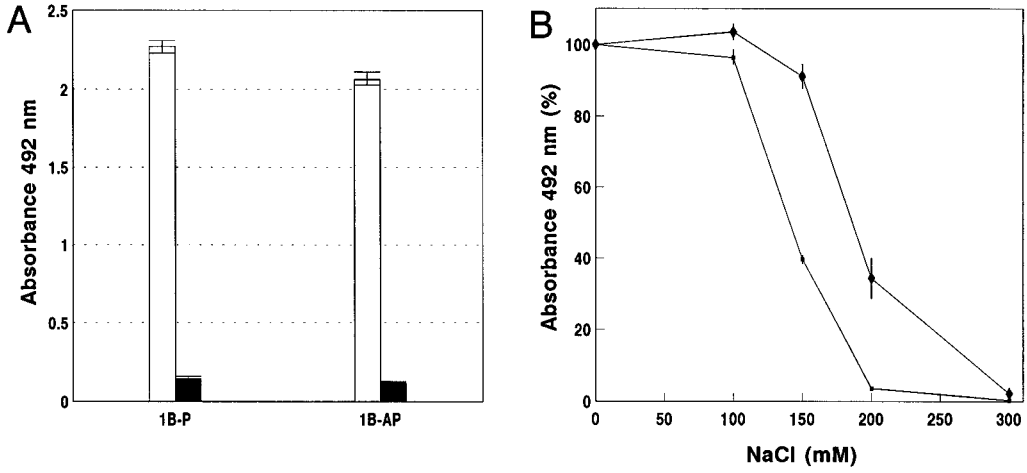

FIGURE 9: Solid phase immunoassay: binding of 1B-P and 1B-AP to taxol-stabilized microtubules. Solid phase immunoassay was performed according to Pedrotti et al. (1994a). Microtiter plate wells coated with 100 μg of taxol-stabilized microtubules/mL were incubated with 30 μg of 1B-P or 1B-AP/mL either in the presence or in the absence of the indicated concentrations of NaCl. Bound MAP1B was detected with anti MAP1B-4 monoclonal antibody. (A) Signal (□) and background (■) for bound 1B-P and 1B-AP in absence of NaCl, respectively. (B) Binding of 1B-P (■) and 1B-AP (◆) to taxol-stabilized microtubules in presence of indicated concentrations of NaCl. Absorbance at 492 nm is expressed as a percentage of MAP1B binding in the absence of salt. Values are a mean of 3−4 determinations.

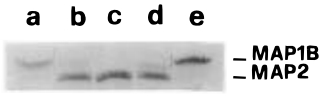

FIGURE 10: Competition between MAP1B and MAP2 for the microtubule binding site. A 60 μg amount of taxol-stabilized microtubules was incubated with 18 μg of 1B-P (lanes a and b) or 1B-AP (lanes d and e) either in the presence (lanes b and d) or absence (lanes a and e) of 18 μg of MAP2. Pellets were fractionated by SDS−PAGE on a gradient (4%−15%) acrylamide gel. Taxol-stabilized microtubules sedimented only in the presence of 18 μg of MAP2 are also shown (lane c).

PDPK sites is phosphorylated: the site for antibody 150 is phosphorylated as this antibody recognizes the protein before but not after AP-treatment, while the site for antibody 531 is not phosphorylated as it recognizes the protein strongly both before and after AP-treatment. Interestingly, the epitope(s) of antibody 150 is the first site to be dephosphorylated upon AP-treatment while the two CKII sites are dephosphorylated at a slower rate, with 842 epitope(s) being the most resistant to AP-treatment. Both PDPK and CKII sites are highly phosphorylated in developing brain where axonal growth occurs, but in adult brain regions cytosolic MAP1B remains phosphorylated in CKII sites and is dephosphorylated in PDPK sites (Ulloa et al., 1993a). The phosphorylation pattern observed would therefore suggest that our preparations may be enriched in late development stage MAP1B.

Phosphorylation of MAPs alters their interaction with microtubules and influences microtubule assembly and dynamics (Burns et al., 1984; Tsuyama et al., 1987). However, unlike MAP2, where phosphorylation weakens its binding to microtubules, phosphorylation of MAP1B by casein kinase II has been suggested to increase its binding to microtubules (Diaz-Nido et al., 1988; Ulloa et al., 1994b). Our observations suggest that dephosphorylation of the PDPK site leads to a large change in mobility and an increase in the affinity of binding to microtubules. On the other hand, dephosphorylation of the CKII sites also results in a change in mobility, albeit much smaller than for the PDPK site, but does not significantly affect the microtubule binding affinity. The primary sequence for MAP1B contains seven sites that are strongly predicted to be phosphorylated by CKII (Noble et al., 1989). Thus taken together, these data suggest that the interaction of MAP1B with microtubules may be regulated by phosphorylation of specific sites either negatively, e.g., epitope(s) of antibody 150, leading to a weakening of affinity for microtubules, or positively by increasing the binding to microtubules in the case of CKII phosphorylation as suggested by Ulloa et al. (1994b), although the CKII phosphorylation sites involved remain unidentified. A similar mechanism has been previously proposed for MAP2 (Brugg & Matus, 1991).

Immunoblotting of proteolytic fragments of MAP1B has shown that the epitopes of antibodies 125, 531, and AA6 reside in the large N-terminal fragment while those for antibodies 842 and 150 reside in the small C-terminal fragment (Ulloa et al., 1994b). The N-terminal fragment has also been shown to contain the microtubule and light chain binding domain (Muller et al., 1994). The observation that dephosphorylation of the epitope(s) for antibody 150 leads to an increase in microtubule binding affinity while that of 125 does not would therefore suggest that phosphorylation-induced conformational changes rather than introduction of negative charges in the binding region cause alterations in microtubule binding affinity. However, phosphorylation/dephosphorylation of N- or C-terminal domains of the heavy chain does not alter its interaction with the light chains.

It has been a generally accepted belief that MAP1B has a lower affinity for binding to microtubules compared with MAP2 [see Muller et al. (1994)]. This notion is derived from observations that (1) MAP1B, in the presence of other MAPs, cycles inefficiently with microtubules in vitro, (2) MAP2 can efficiently displace MAP1B from microtubules (Joly & Purich, 1990; Pedrotti & Islam, 1995), and (3) drug resistance experiments with Nocodazole using transfected cell lines (Takemura et al., 1992) show that MAP2 and τ are more efficient stabilizers of microtubules compared with MAP1B. This is further confirmed by the observed ability of MAP2 to displace MAP1B, both in its phosphorylated and dephosphorylated states, from binding to microtubules and may suggest similar or overlapping sites on the tubulin molecule for both MAPs. Alternatively, the higher affinity of MAP2 compared with MAP1B may reflect differences in the manner in which the two proteins interact with the microtubule. Indeed, the relative stoichiometries of MAP2 and MAP1B are different and the MAP1B microtubule binding site composed of KKE sequences may be helical (Avila, 1991), while the MAP2 microtubule binding repeats contain helix breakers (Lewis et al., 1988). The "helix−helix" interaction of MAP1B:tubulin may screen the electrostatic repulsion between tubulin dimers differently when compared with the "linear-helix" interaction of MAP2:tubulin; association of tubulin dimers with each other requires a screening of negative surface charges (Pedrotti et al., 1994a). The lower affinity of binding, and probably lower stabilization of microtubules (Takemura et al., 1992), suggests that MAP1B may give rise to much more dynamic microtubules than those observed with the classical MAPs, e.g., MAP2 and τ. Indeed, MAP1B is one of the earliest MAPs to appear during development and is concentrated in the distal region of the growing axon, regions which represent major sites of microtubule assembly dynamics (Brown et al., 1992; Black et al., 1994; Ulloa et al., 1994a).

A comparison of properties exhibited by MAP1B and MAP1A is also interesting. MAP1A, like MAP1B, also contains KKE motifs, but, unlike MAP1B, MAP1A colocalizes on the same microtubules with MAP2, suggesting that it binds to different binding sites (Pedrotti & Islam, 1994; Cravchik et al., 1994). Indeed, Cravchik et al. (1994) have recently identified a novel acidic microtubule binding domain in MAP1A. MAP1A shows another property in common with MAP2 in that it binds to and cross-links actin filaments while MAP1B fails to bind to G- or F-actin under similar assay conditions (Pedrotti et al., 1994b; Pedrotti & Islam, 1995). MAP1A and MAP1B, however, behave as heat-labile proteins in crude brain supernates but are heat-stable when pure, whereas MAP2 is heat-stable under both of these conditions. Thus, despite their sequence and structure similarities MAP1A and MAP1B behave quite differently biochemically.

The MAP1 proteins exhibit a complementary pattern of expression during brain development. The differences in microtubule assembly behavior between the MAP1 proteins may be important in cells during the transformation changes from a "plastic" to a more "stable" stage in neuronal processes. It is noteworthy that MAP1B exhibits concentra-

tion gradients being preferentially concentrated in the distal region of the growing axon and continues to be present in significant amounts in areas with a high neurogenerative capacity.

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