Specific Binding of Acidic Phospholipids to Microtubule-associated Protein MAP1B Regulates Its Interaction with Tubulin*

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Microtubule-associated protein MAP1B, a major neuronal cytoskeletal protein, is expressed highly during the early stage of brain development and is thought to play an important role in brain development. Although it has been shown that MAP1B localizes both in cytosol and particulate fractions, the underlying molecular mechanism in the membrane localization has yet to be elucidated. In the present study, we show that MAP1B purified from young rat brain can bind to acidic phospholipids, such as phosphatidylserine, but not to a neutral phospholipid, phosphatidylcholine. Furthermore, the binding of MAP1B to taxol-stabilized microtubules was inhibited by the addition of phosphatidylserine or phosphatidylinositol. The addition of phosphatidylinositol showed no effect on the binding of MAP1B to the microtubules. A 120-kDa microtubule-binding fragment of MAP1B was also released from microtubules by the addition of acidic phospholipids. Synthetic peptides derived from the C-terminal half of the tubulin-binding domain, but not that corresponding to the N-terminal half, bound to acidic phospholipids specifically. These results suggest that MAP1B binds to biological membranes through its tubulin-binding site, and the binding may play a regulatory role in MAP1B-microtubule interaction.

Microtubules play an important role in cell organization and a variety of cell functions such as growth, proliferation, and differentiation. Microtubule-associated proteins (MAPs)\(^1\) are thought to regulate the formation and stability of the microtubules. Protein phosphorylation of MAPs is known to alter their interaction with tubulin and to affect the stability of the microtubule cytoskeleton. Mitogen-activated protein kinase, for example, has been initially purified as a protein kinase that phosphorylates MAP2, and the involvement of mitogen-activated protein kinase-dependent phosphorylation in the reorganization of the cytoskeleton after various cellular stimuli has been well established (1). The cytoskeletal proteins including the MAPs are, in fact, one of the major targets of the signaling pathways. Phosphorylation of the MAPs plays important roles not only in the physiological functions but also in pathological aspects. For example, tau protein that accumulates in paired helical filaments, pathological structures that develop within neurons in Alzheimer’s disease, is aberrantly phosphorylated (2–4). Phosphorylated MAP1B is also found in paired helical filaments (5).

MAP1B (microtubule-associated protein 1B, also known as MAP1X, MAP1.2, or MAP5) is a component of neuronal cytoskeleton in developing brain or in the regenerating region of adult brain such as the olfactory bulb (6–8). Although MAP1B is classified as a MAP, MAP1B is different from other MAPs in several respects. While MAP1B lacks the microtubule-binding motifs common to MAP2, MAP4, and tau protein, it contains a unique microtubule-binding domain composed of multiple KX(E/I/V) repeats (9). Furthermore, MAP1B shows relative inefficiency in microtubule co-sedimentation (6). Although the physiological function of MAP1B has yet to be elucidated, its phosphorylation has been suggested to be important for synaptogenesis and synapse formation. Phosphorylation by casein kinase II has been proposed to be required for neuritogenesis (10). MAP1B is strongly phosphorylated in cultured cortical neurons undergoing synaptogenesis, and the inhibition of the phosphorylation of the protein results in the suppression of the synapse formation (11). These observations suggest that the phosphorylation of MAP1B plays an important role. Recent data from gene targeting experiments in mice indicate that MAP1B is necessary for normal development of the central nervous system (12), although the molecular basis of abnormalities seen in the MAP1B-deficient pups has yet to be elucidated.

Protein phosphorylation is not the only regulatory means of the MAP functions. Phosphatidylinositol has been shown to bind specifically to MAP2, and the binding affects the interaction between MAP2 and tubulin (13–15). MAP1B is suggested to be localized both in cytosolic and particulate fractions (16). It is also known that MAP1B is localized to the particular fraction of the growth cone (17). MAP1B of these two fractions shows different immunostaining with several antibodies that recognize different epitopes of phosphorylated MAP1B, suggesting that the MAP1B localization may be regulated by its phosphorylation. Although it has been speculated that the localization to a particular fraction of MAP1B may be due to the association to microfilaments, no molecular basis for the membrane localization of MAP1B has been presented. In the present study, we show that purified MAP1B can bind specifically to acidic phospholipids. The binding of acidic phospholipids to the C-terminal half of the tubulin-binding domain induced MAP1B dissociation from taxol-stabilized microtubules, suggesting that acidic phospholipids may play a regulatory role in the MAP1B function.

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\(¶\) The abbreviations used are: MAP, microtubule-associated protein; PC, phosphatidylcholine; PA, phosphatic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP\(_2\), phosphatidylinositol 4,5-diphosphate; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

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**EXPERIMENTAL PROCEDURES**

**Materials**—MAP1B was purified from the cytoplasmic fractions of young rat brain as described (18) with some modifications. The whole brain was homogenized in 100 mM PIPES-NaOH buffer (pH 6.8) containing 2.5 mM EGTA, 0.5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 40 μg/ml leupeptin, and 1 μg/ml pepstatin and centrifuged at 100,000 × g for 60 min. After adding 20 μl paclitaxel, the supernatant was incubated at 25°C for 60 min. Assembled microtubule proteins were sedimented by centrifugation at 100,000 × g for 60 min. The supernatant containing most of the MAP1B protein was adjusted to 0.2 M NaCl and applied onto a Resource 15Q column (1 × 15 cm; Pharmacia Biotech Inc.). Bound proteins were eluted by a 0.2–1 M NaCl gradient in 100 mM MES-NaOH buffer (pH 6.4) containing 2.5 mM EGTA, 0.1 mM EDTA, 0.5 mM MgCl₂, and 1 mM dithiothreitol (MES buffer).

MAP1B-containing fractions were pooled, diluted, and applied to a Mono S column (HR 5/5, Pharmacia), and the column was eluted by a 0–1 M NaCl gradient in MES buffer. MAP1B thus purified was stored at −80°C until use. Tubulin was prepared from bovine brain as described (19). Phospholipids were purchased from Avanti Polar Lipids. Phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-diphosphate (PIP₂) were obtained from Sigma. Monoclonal AA6, HM-1, and E12 antibodies were purchased from Sigma, while monoclonal anti-MAP2 antibody was purchased from Chemicon International Inc. Peroxidase-linked anti-mouse IgG was purchased from Amersham Corp. All other chemicals used were of analytical grade.

**Preparation of Phospholipid Vesicles**—Phospholipids in chloroform solution were dried to a thin film under a stream of argon gas and kept under vacuum for 30 min. The lipid films were dispersed in a 170 mM sucrose solution containing 1 mM Tris-HCl buffer (pH 7.5), followed by 10 cycles of extrusion through a stack of polycarbonate filter (0.1-μm pore size) in a LiposoFast-Basic apparatus (AVESTIN). Sucrose was removed from the solution outside the vesicles by dialyzing the vesicle solution 5-fold with 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and by centrifugation at 100,000 × g at 25°C for 60 min. Pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and used as large unilamellar vesicles. Small unilamellar vesicles were made by sonication using a tip-type sonicator (Branson Sonifier 250) for 30 min as described (20). After centrifugation in a table top centrifuge for 20 min, supernatants were used as small unilamellar vesicles.

**Sedimentation Assay**—Binding of MAP1B and that of the MAP1B-derived peptides to phospholipid membranes were analyzed by sedimentation analysis. MAP1B (2.5 μg) was mixed with large unilamellar vesicles (5 μg) in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and incubated at 30°C for 30 min. After centrifugation at 200,000 × g for 60 min, protein co-sedimented with vesicles was analyzed by SDS gel electrophoresis (20). Peptides (10 μg) were mixed with PS or PC vesicles (2.4 mg) in 60 μl of 100 mM PIPES-NaOH buffer (pH 7.5) containing 100 mM NaCl and incubated at 30°C for 30 min followed by centrifugation at 200,000 × g for 60 min. Peptides remaining in supernatants were analyzed by SDS gel electrophoresis.

**Microtubule Binding Assay**—MAP1B (7.5 μg) was mixed with paclitaxel-stabilized tubulin (0.2 mg) and digested with chymotryptic cleavage of MAP1B as described previously (8). MAP1B (5 μg) was mixed with paclitaxel-stabilized tubulin (0.2 mg) and the membranes were immunostained with monoclonal antibodies against MAP1B, MAP2, MAP1A, and LC1 using ECL (Amersham). Protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard.

**RESULTS**

**Purification and Characterization of MAP1B from Rat Brain**—MAP1B was purified from cytosolic fractions of young (1-week-old) rat brain after sedimenting most of the microtubule-associated proteins other than MAP1B as microtubules (18). Although GTP- and temperature-dependent polymerization of microtubules in the original procedure was substituted with paclitaxel-dependent polymerization, most of the MAP1B protein remained in the supernatant and could be purified to an apparent homogeneity after two successive column chromatography steps (Fig. 1a). The purified protein contained one heavy chain of around 250 kDa and two light chains of 30 (LC1) and 15 kDa (LC3). The presence of LC3 was seen only when the gel was overloaded (data not shown). Purified MAP1B was stained in the immunoblot only by the anti-MAP1B monoclonal antibody, and no significant staining was observed with anti-MAP1A or anti-MAP2 antibody, suggesting that the purified MAP1B was free from other MAPs of similar molecular weights. In addition, MAP1B preparation was also stained with anti-MAP1 light chain antibody (Fig. 1b). The identity of

2 Residue numbers refer to the combined amino acid sequence deduced from two cDNA sequences (23, 24) with one exception. Although Arg355 found in the mouse sequence is missing in the original rat sequence, our mass spectrometric analysis indicated the presence of an Arg in the position (data not shown).

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**FIG. 1. SDS gel electrophoresis and immunoblot of MAP1B purified from rat brain.** A, SDS gel electrophoresis of MAP1B isolated from young rat brain. A 5–20% acrylamide gradient gel was used. Western blot of purified MAP1B. The blotted membrane was immunostained using AA6 (anti-MAP1B, lane 1), E12 (anti-MAP1 light chain, lane 2), MAB364 (anti-MAP2, lane 3), or HM-1 (anti-MAP1A, lane 4) antibodies.
the purified protein as MAP1B was further confirmed by mass spectrometric analysis. The purified protein was digested with lysyl endopeptidase, and the resulting peptide mixture was subjected to liquid chromatography/electrospray mass spectrometry (21, 22). As will be described elsewhere,3 more than 70 peptides could be assigned to the sequence deduced from the published cDNA sequence of rat MAP1B (23, 24), establishing the identity of the protein.

**Binding of MAP1B to Phospholipid Membranes**—To examine whether MAP1B can interact with membrane phospholipids, binding of MAP1B to phospholipid vesicles was directly studied by sedimentation assay. When purified MAP1B was incubated with large unilamellar vesicles of phosphatidylserine (PS), phosphatidylcholine (PC), or phosphatidic acid (PA) and subjected to centrifugation, similar bindings were observed with all of the acidic phospholipids used, demonstrating that MAP1B has an intrinsic ability to bind to phospholipid membranes (Fig. 2). Similar binding was observed with phospholipid vesicles containing a mixture of PC (80%) and PS (20%) that mimics that of biological membranes such as the cytoplasmic leaflet of typical mammalian plasma membranes (data not shown). On the contrary, no significant binding was observed when phosphatidylcholine (PC), a neutral phospholipid, was used. These results suggest that the ionic interactions rather than the hydrophobic interactions play an important role in the MAP1B-membrane interaction.

**Effects of Phospholipids on MAP1B-Tubulin Interaction**—Since the main physiological function of MAP1B is in the interaction with microtubules, the effects of phospholipids on the MAP1B-tubulin interaction were studied. Purified rat MAP1B was found in the pellets when paclitaxel-stabilized tubulin was sedimented by centrifugation (Fig. 3a, lanes 1 and 2), demonstrating that the purified MAP1B has the ability to bind to microtubules. The addition of PC, which did not show significant binding to MAP1B, to the mixture did not show any effect on the MAP1B-tubulin interaction as expected (lanes 3 and 4). However, when acidic phospholipids were included in the mixture, MAP1B did not co-sediment with tubulin but remained in the supernatants (lanes 5–10). Under the experimental conditions employed, most of the lipids remained above the 50% sucrose cushion, while the polymerized tubulin was found in the pellets. Therefore, the addition of acidic phospholipids induced dissociation of MAP1B from the polymerized tubulin. The formation of a ternary complex among the phospholipid membranes, MAP1B, and tubulin was not observed, suggesting either that the binding of acidic phospholipids to MAP1B induces a conformational change that results in the loss of binding to tubulin or that the acidic phospholipids share the same binding site(s) in MAP1B with tubulin. PI (lanes 9 and 10) and PS (lanes 7 and 8) seem to be more effective than PA (lanes 5 and 6) in the dissociation of MAP1B from polymerized tubulin. The order of acidic phospholipid addition prior to or after the binding of MAP1B to tubulin did not change the observed behavior of MAP1B release (Fig. 3b, lanes 1–4).

Phosphatidylinositol is known to be metabolized further to various messengers upon cellular stimulation. It is, therefore, of interest to test the effects of phosphatidylinositol phosphates on the MAP1B-tubulin interactions. Two acidic metabolites of PI, i.e. phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-diphosphate, were tested for their abilities to bind MAP1B as well as to affect the MAP1B-tubulin interaction. Under the same experimental conditions, MAP1B bound to PI completely but not to phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-diphosphate to a significant extent (data not shown). The addition of the phosphatidylinositol phosphates did not affect the MAP1B-microtubules interaction significantly, while PI inhibited the interaction effectively (Fig. 3c, lanes 1–4). These results demonstrate that the interaction between MAP1B and phospholipids is not affected by simple ionic interactions but involves specific structural interactions.

Assuming a molecular weight of 300,000 for MAP1B and

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3 E. Yamauchi, K. Muramoto, Y. Kuroda, and H. Taniguchi, manuscript in preparation.
100,000 for tubulin dimer, the concentrations of the proteins used in these binding assays were 0.3 μM for MAP1B and 24 μM for tubulin. These values were chosen from their concentrations in vivo; tubulin constitutes around 13–25% of total protein in brain, and the molar concentration can be calculated to be 20–50 μM (25, 26), while the amount of MAP1B in neonatal brain has been estimated to be around 0.35 μM (27). Therefore, the interaction of MAP1B with acidic phospholipids seems to occur at physiologically relevant concentrations. We performed a similar binding assay with a series of MAP1B and tubulin concentrations at a fixed PS concentration (Fig. 4). When the protein concentrations were lowered by a factor of 5, about 75% of MAP1B was found in the supernatant with the phospholipid (Fig. 4, lane B). However, most of MAP1B remained associated with tubulin when the protein concentrations were further reduced to one-tenth of the original concentrations. These results not only indicate that the acidic phospholipids have high affinities to MAP1B but also suggest that the tubulin-MAP1B interaction can be reversibly regulated by acidic phospholipids. This raises the possibility that the acidic phospholipids play a regulatory role in the tubulin-MAP1B interactions in cells.

The Phospholipid-binding Site Is Localized within the Tubulin-binding Site of MAP1B—To identify the phospholipid-binding domain, MAP1B was treated with chymotrypsin, which has been shown to produce a 120-kDa microtubule-binding fragment, which corresponds to the N-terminal half of the molecule (8). Effects of PS on the 120-kDa microtubule-binding fragment-tubulin interaction were then studied in the same way as above. The fragment behaved similarly to the intact MAP1B; the addition of PS vesicles to a complex of the 120-kDa microtubule-binding fragment and tubulin induced the dissociation of the fragment from microtubules, while PC had no effect on the interaction (Fig. 5).

Since the phospholipid binding to MAP1B facilitates its dissociation from tubulin, the simplest explanation is that phospholipids and tubulin share the same binding site in MAP1B. To test this hypothesis, three peptides corresponding to the different parts of the microtubule-binding domain were designed as described under “Experimental Procedures” (see also Fig. 7). The rationale of the present design of peptides is as follows. Previous deletion and recombination studies (9) demonstrated that the “domain X” between amino acids 646 and 732 can bind to tubulin, but a deletion mutant lacking the domain can still bind to tubulin. Therefore, another domain either N- or C-terminal to domain X constitutes a second tubulin-binding site. The typical KKE(E/I/V) tubulin-binding motif is clustered in domain X, while the domain C-terminal to it contains fewer KKE(E/I/V) motifs, and each motif becomes less characteristic. The overall effect is that the domain C-terminal to domain X contains fewer acidic residues, the net charge becomes more positive, and the content of hydrophobic amino acid increases. Therefore, peptide 1 was chosen from the central part of domain X, and peptide 3 was chosen from the C-terminal half of the tubulin-binding domain. Peptide 2 is adjacent to peptide 3 and similar to the latter, but it still retains one typical KKEE motif. It also contains fewer basic residues compared with peptide 3. This gives the peptide a character that is intermediate between the two other peptides. When these peptides were mixed with PS or PC vesicles, three peptides showed different affinities to phospholipids (Fig. 6). Peptide 1 did not show any significant binding either to PC or PS. On the contrary, peptides 2 and 3 bound to PS vesicles specifically. No significant binding to PC vesicles was observed with all of the peptides examined. Thus, the C-terminal half of the microtubule-binding domain of MAP1B, but not the N-terminal half containing typical KKE(E/I/V) repeats, has the ability to bind to acidic phospholipids specifically. These results suggest that the C-terminal half of the microtubule-binding domain of MAP1B serves as a membrane or acidic phospholipid-binding domain, and the binding of lipids decreases the MAP1B-tubulin interaction.
MAP1B is a major MAP in nerve growth cone and is enriched in its membrane-cytoskeleton fraction (17). Since MAP1B is a very hydrophilic protein without any apparent hydrophobic membrane-binding sites, either direct binding to other proteins such as actin filaments (28) is involved in the localization or the protein binds to the membranes through a domain(s) that is not simply hydrophobic. The present study established that MAP1B has an intrinsic ability to bind to acidic phospholipids and that a subdomain with a basic amphiphilic nature within the microtubule-binding domain is responsible for the binding. Furthermore, we found that the binding of acidic phospholipids to the protein affects its interaction with tubulin.

A hydropathy plot of MAP1B sequence shows a single hydrophobic domain between residues 791 and 817 that is located just C-terminal to the tubulin-binding domain (11). However, the apparent high hydrophobicity of the region is mainly due to the lack of hydrophilic charged amino acids, and the domain lacks large hydrophobic residues (Fig. 7). Since MAP1B binds to acidic phospholipids rather than to neutral phospholipids, such a neutral domain cannot be the primary binding site. On the contrary, a closer look of the adjacent tubulin-binding site revealed an interesting variation of the character within the domain. The N-terminal half of the domain consists mainly of the typical KKE(E/I/V) motif, while the C-terminal half contains a derivative of the motif that lacks the acidic residues. This gives the latter domain a basic amphiphilic character, in which basic residues and hydrophobic residues appear alternatingly.

This is reminiscent of the membrane-binding domains, which have been identified in various proteins including MARCKS (29), GAP-43 (30), nitric-oxide synthase (20), and pp60\(^{c-src}\) (31). In fact, such a basic amphiphilic domain seems to be a common membrane-binding motif in a variety of proteins (32, 33). It is of interest to note that the membrane interactions of these domains are often regulated by protein phosphorylation (20, 28). Since MAP1B is known to be phosphorylated at multiple sites (16) and the interactions of other MAPs such as tau and MAP2 with tubulin are regulated by protein phosphorylation of the binding domains (34, 35), this raises an intriguing possibility that such a regulatory mechanism is operational in the MAP1B-tubulin interactions.

MAP1B is the first MAP expressed highly during development of the brain. The level of its expression, however, decreases rapidly during synaptogenesis with a concomitant increase in the MAP1A expression level. Both proteins share a high homology, especially at the N-terminal half of the molecules including the N-terminal half of the tubulin-binding domain (36). On the other hand, the C-terminal half of the MAP1B tubulin-binding site, which is identified as the phospholipid-binding site in the present study, is lacking in the MAP1A sequence. It is of interest to note in this context that the MAPs fractions prepared from adult bovine brain containing mostly MAP1A, MAP1C, and MAP2 behave differentially upon addition of acidic phospholipids. Namely, only MAP2 but not MAP1 dissociates from microtubules (13). This corresponds well with the lack of the phospholipid-binding site in the MAP1A tubulin-binding domain. The difference may be important for the functional difference between MAP1A and MAP1B.

As mentioned above, MAP1B is not the only MAP whose function is regulated by the specific binding of phospholipids. MAP2C and tau have been shown to bind acidic phospholipids such as phosphatidylinositol with a high affinity (15). Many actin-binding proteins also bind to phospholipids, and their functions are regulated by the specific binding as well (37). Developing neurons, especially at their growth cone, should undergo dynamic rearrangements of membrane-cytoskeleton, and phospholipid-binding cytoskeletal proteins, which now include MAP1B, may play important roles in these events.

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