The Microtubule-binding Fragment of Microtubule-associated Protein-2: Location of the Protease-accessible Site and Identification of an Assembly-promoting Peptide

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Abstract. Thrombin cleavage of bovine brain microtubule-associated protein (MAP-2) yields two stable limit polypeptide fragments (28,000 and 240,000 Mr). The smaller cleavage product contains the microtubule-binding domain and is derived from the carboxyl terminus of MAP-2 while the 240,000 Mr fragment is derived from the amino terminus. The amino terminal sequence of the smaller cleavage product is homologous with the microtubule-binding fragment of tau in sequence and in a similar location relative to three imperfect octadecapeptide repeats implicated in microtubule binding. Peptides corresponding to the cleavage site and the three repeats of MAP-2 were synthesized. Only the second octadecapeptide repeat (VTSKCGSLKNIRHRPGGG) was capable of stimulating microtubule nucleation and elongation. Microtubules formed in the presence of this peptide displayed normal morphology and retained the inhibition properties of calcium ion, podophyllotoxin, and colchicine. Our result indicates that a region comprising only ~1% of the MAP-2 sequence can promote microtubule assembly.

Considering the wide variety of microtubule functions, the need for temporal, spatial, and metabolic control of microtubule assembly/disassembly is quite evident (Purich and Kristofferson, 1984; Olmsted, 1986). In this regard, it is not surprising that there should be a large number of proteins, beyond tubulin per se, that influence microtubule self-assembly. Such proteins are collectively known as microtubule-associated proteins (MAPs), and they include the MAP-1, MAP-2, and tau protein families. The designation of proteins as MAPs has been based operationally on their ability to purify with assembled microtubules through cycles of warm-induced assembly and cold depolymerization. More recently, however, sequence data on the MAP-2 and tau proteins suggest that they share a microtubule-binding motif (Lewis et al., 1988), and three imperfectly repeated sequences have been postulated to constitute the microtubule-binding site. Aizawa et al. (1988) also observed that chymotrypsin cleavage of tau occurs at a specific proline-rich sequence, defining the NH₂ terminus of the microtubule-binding fragment in the tau proteins. We have investigated the site of thrombin cleavage of MAP-2 because our earlier studies had shown that this Mr 28,000 thrombin cleavage fragment binds to both microtubules and neurofilaments (Flynn et al., 1987). We now report that thrombin attacks a region of MAP-2 that corresponds to the site of chymotrypsin cleavage of tau. Furthermore, by using oligopeptides corresponding to the NH₂ terminus and the three imperfect repeats in the MAP-2 binding fragment, we have explored the ability of such peptides to stimulate microtubule assembly. Our results suggest only the peptide corresponding to the second repeat can promote nucleation and elongation of microtubules.

Materials and Methods

Materials

[35S]ATP (7,000 Ci/mmol) and [3H]GTP (18 Ci/mmol) were purchased from ICN (ICN Radiochemicals, Irvine, CA) along with ultrapure grades of ammonium sulfate, SDS, acrylamide, and bis-acrylamide (ICN Biomedicals, Irvine, CA). Acetate kinase was a product of Boehringer Mannheim Biochemicals (Indianapolis, IN), while phosphocellulose resin was from Whatman Inc. (Clifton, NJ). Immobilon was obtained from Millipore Continental Water Systems (Bedford, MA). DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ), and bovine thrombin, trifluoracetic acid, Mes buffer, and PMSF were from Sigma Chemical Co. (St. Louis, MO). t-BOC amino acids and phenylacetylmethyl resin were from Applied Biosystems Inc. (Foster City, CA).

Preparation of Proteins

Bovine brain microtubule protein was prepared by the procedure of Shelanski et al. (1973). MAP-2 was purified by the method of Herzog and Weber (1978) and radiolabeled as previously described (Flynn et al., 1987). Phosphocellulose purified tubulin was prepared according to Kristofferson et al.
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Phosphate (Sigma Chemical Co.), and 0.1 mM [3H]GTP (20 Ci/ml). All assay mixes also contained 1 mM [YrT. Peptides were weighed out just before use and dissolved in 100 mM Pipes, pH 6.8, 1 mM EGTA, and 1 mM magnesium sulfate at 4°C with 1 mM PMSF and passed over a 1 ml DEAE-Sephadex A-50 column equilibrated in the same buffer. The breakthrough fractions were pooled and precipitated with 60% (wt/vol) ammonium sulfate. After sedimentation, the precipitate was resuspended in assembly buffer and used for HPLC analysis or Immobilon blotting.

HPLC and Sequence Analysis

HPLC was carried out on a chromatograph (model 1090A; Hewlett-Packard Co., Palo Alto, CA), equipped with a diode array detector. The protein was loaded on a C-18 column (Waters Associates, Milford, MA) equilibrated in 0.1% (vol/vol) trifluoroacetic acid, and eluted with a linear gradient of 0-50% (vol/vol) acetonitrile with 0.1% (vol/vol) trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractions containing the MAP-2 microtubule-binding domain were pooled, dialyzed against 100 mM ammonium bicarbonate, and lyophilized.

Immobilon was handled according to the manufacturer's instructions before electroblotting. A 12% acrylamide SDS-gel containing heat-stable microtubule-binding fragments was electrophoretically transferred to the membrane for 6 h at 70 V in 10 mM 3-[cyclohexylamino]-l-propane-sulfonate, pH 10.0, with 10% methanol. The membrane was stained with Coomassie brilliant blue R-250, destained in 50% methanol-10% acetic acid, and the band of interest excised with a razor blade and sequenced in a gas-phase protein sequencer (model 470A; Applied Biosystems Inc.) with on-line phenylthiohydantoin analyzer. Ultrapure grades of SDS, acrylamide, and bis-acrylamide were used to avoid blocking the NH2 terminus.

In the absence of thrombin treatment, identical sequence experiments with either electroblotted MAP-2, as well as MAP-2 in solution, did not yield any phenylthiohydantoin derivatized amino acids at detectable levels. Likewise, experiments with the immobilized 240,000-Mr, projection-arm fragment yielded no sequence data. Protein samples failing to yield detectable levels of amino acid derivatives were subjected to acid-catalyzed hydrolysis and amino acid analysis to assure that sufficient levels of protein for sequencing had been employed. These findings suggest that the amino terminus of MAP-2 is blocked and that the 240,000-Mr fragment lies at the amino terminus whereas the 28,000-Mr fragment resides at the carboxyl end. These observations are in accord with the findings by Kosik et al. (1988) who reported that the NH2 terminus of MAP-2 appears to be blocked.

Microtubule Assembly with Synthetic Peptides

All assembly experiments were done with a GTP-regenerating system (Mac-Neal et al., 1977) consisting of 2 U/ml of acetate kinase, 10 mM acetyl phosphate (Sigma Chemical Co.), and 0.1 mM [3H]GTP (20 Ci/ml). All assay mixes also contained 1 mM DTT. Peptides were weighed out just before use and dissolved in 100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM magnesium sulfate with 1 mM DTT. Varying concentrations of each peptide were added to 1.6 mg/ml pure tubulin and 0.4 mg/ml three cycle microtubule-protein and incubated at 30°C for 30 min. The extent of microtubule assembly was monitored by the rapid filtration assay of Maccioni and Seeds (1978) as modified by Wilson et al. (1982). Microtubules were diluted 20× into 100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM magnesium sulfate, 1% glutaraldehyde (Sigma Chemical Co.), 10% dimethylsulfoxide, 25% glycerol, and 1 mM ATP and kept at 30°C until ready to assay. The diluted mixture was then applied to GPF filters on a vacuum filtration device (Hoeffer Scientific Instruments, San Francisco, CA) presoaked in the same buffer except no glutaraldehyde was used. Each filter was then washed with 15 ml of the same buffer and the radioactivity was solubilized in 1.5 ml 0.1 N NaOH for 30 min followed by addition of scintillation cocktail (Research Products International Corp., Mt Prospect, IL).

Preparation of Microtubule Seeds and Elongation Assay

Microtubule fragments or seeds were prepared according to Kristofferson et al. (1986). Tubulin at 5 mg/ml was assembled with 1 mM GTP in 80 mM Pipes, pH 6.8, 10 mM magnesium chloride, 1 mM EGTA, in 30% glycerol at 37°C for 30 min. The microtubules were then sheared with three passes through a 22-gauge needle to produce microtubule seeds. The seeds were diluted 100× (vol/vol) into solutions containing 0.5 mg/ml tubulin at varying concentrations of peptides. After 30 min at 37°C, the samples were handled as described in the preceding section for measurement of radioactive guanine nucleotide incorporation.

Results

Thrombin Cleavage of MAP-2 and Purification of Its Microtubule-Binding Fragment

Vallee (1980) first demonstrated that MAP-2 can be fragmented into 35,000- and 240,000-Mr components by chymotrypsin or trypsin. The smaller fragment contains the microtubule-binding domain, and the larger is designated as the projection-arm domain. While these protease cleavage products have been very useful in many investigations of microtubule self-assembly, chymotryptic and tryptic cleavage do not yield stable limit polypeptides. Because thrombin is an arginine-specific serine protease, we reasoned that thrombin might display a simpler fragmentation pattern. MAP-2 cleavage can be readily assessed by SDS gel electrophoresis of [32P]MAP-2 because this protein is extensively phosphorylated (Theurkauf and Vallee, 1983). The projection-arm fragment contains many more phosphoryl acceptor sites than the tubule-binding domain; even so, the stability of the 28,000-Mr fragment is evident in the autoradiogram presented in Fig. 1. This thrombin-produced fragment of MAP-2 possesses the microtubule-binding site as well as a neurofilament-binding site as previously reported (Flynn et al., 1987).

Amino-Terminal Sequence of the Microtubule-Binding Fragment

To gain more information about this site of facile thrombin cleavage, we developed a high-yield isolation method (see Materials and Methods) for amino acid analysis and sequencing experiments. We employed protein microsequencing techniques with the 28,000-Mr fragment electroblotted from SDS-polyacrylamide gels to a derivatized nylon screen (Immobilon). We obtained the amino terminal sequence shown in Fig. 2. Lewis et al. (1988) reported the entire derived amino acid sequence using murine MAP-2 cDNA clones. Our primary sequence data with bovine brain MAP-2 correspond to the murine sequence spanning residues 1626-1644 with only three exceptions.

As shown in Fig. 2, there is a similar protease-accessible sequence in the microtubule-binding fragment of bovine tau protein. In that case, however, fragments were generated by chymotryptic cleavage (Aizawa et al., 1988). Both of these cleavage-site sequences reside ~40-50 residues toward the NH2 terminal side of the first of three nonidentical octa-
Figure 1. Time course of thrombin cleavage of bovine brain MAP-2 and purification of its microtubule-binding domain. Radiolabeled heat-stable MAP-2 (32p, 50,000 cpn/μg) was incubated at 37°C with 4 U/ml thrombin for the indicated time in minutes. The digestion was quenched by heating at 100°C for 5 min in the presence of SDS, and the products were resolved on a 15% polyacrylamide gel. The gel was then dried under vacuum and exposed (X-AR 5 film; Eastman Kodak Co., Rochester, NY).

decapeptide repeats (indicated schematically by arrowheads) found in both MAP-2 and tau (Lewis et al., 1988; Lee et al., 1988).

Peptide Interactions with Tubulin and Microtubule-Protein

To analyze further sequence(s) responsible for MAP-2 binding to tubulin within the 28,000-Mr fragment, we synthesized four peptides. The first (m1 = TPHTPGTPK) corresponded to the NH₂-terminus of the 28,000-Mr fragment. The others corresponded to the three octadecapeptide repeats (m2 = VKSKIGSTDNIKYQPG; m3 = VTSKCGSLKNNHRPGG; m4 = AQAQGSDLNAHVPGGG). Peptide m1 was based on our bovine sequence data. We used the murine MAP-2 sequence data for m2, m3, and m4 because no such data are yet available for the bovine MAP-2. The high state of purity of each peptide was confirmed on the basis of HPLC elution profile analysis or gas-phase microsequencing.

We sought to determine whether any of these peptides would influence the assembly of microtubule protein that contained both tubulin and MAPs. We first worked with recycled microtubule protein to which sufficient pure tubulin was added to lower the content of MAPs to about one-fifth their normal level. This final composition was 85% MAPs and 95% tubulin by weight. This ratio was chosen to accentuate any stimulatory effects of the peptides on the assembly process, and no microtubule polymerization occurred at the protein concentrations used without peptide addition. To assay the extent of microtubule assembly at different levels of peptides m1, m2, m3, and m4, we measured [3H]guanine nucleotide uptake with the glass fiber filter assay of Maccioni and Seeds (1978) as modified by Wilson et al. (1982). Only peptide m2, corresponding to the second repeat in MAP-2, stimulated microtubule-assembly as evidenced by the data shown in Fig. 3. When peptides m1, m3, or m4 were employed, no incorporation of guanine nucleotide was observed above background levels. Moreover, in companion experiments, we found that none of these peptides altered the stimulation of microtubule assembly by peptide m1. We also tested the action of several common inhibitors of microtubule assembly to learn whether peptide m2 induced assembly in a manner akin to normal assembly of brain microtubules. Inclusion of colchicine (0.1 mM), calcium ion (2 mM), or podophyllotoxin (0.1 mM) resulted in complete inhibition of peptide m2-induced assembly.

We also found that assembly of pure tubulin could be stimulated by m2 only. Indeed, assembly with tubulin and m2 exhibits a typical time-course for the polymerization process as shown in Fig. 4A. Without addition of m2 peptide, no tritium label is retained on the glass fiber filters. We verified that the observed polymerization resulted in microtubules by using EM and immunofluorescence microscopy (data not shown). When tubulin (1 mg/ml) was incubated with and without peptide m2 (1 mM), intact microtubules were observed only in those micrographs of samples to which this peptide had been added. This concentration of tubulin

Figure 2. Comparison of proteolytic fragmentation patterns and the amino terminal sequences of the microtubule-binding fragments of MAP-2 and tau protein. The polypeptide chains and cleavage patterns for MAP-2 and tau are represented as heavy lines. The closed circles represent the blocked MAP-2 NH₂-terminus, and the underlined amino acid residues represent identical and/or conserved amino acid residues common to both MAP-2 and tau proteins. The tau protein scheme is based on the data of Aizawa et al. (1988) and Lewis et al. (1988).
was clearly above the critical concentration for peptide \( m_2 \)-induced assembly, whereas it was near the critical concentration for polymerization of pure tubulin (see Fig. 4 B).

These observations indicate that only the peptide \( m_2 \), with a sequence corresponding to the second repeated region of the microtubule-binding fragment MAP-2 could stimulate tubulin assembly. Nonetheless, the possibility remained that the other peptides could still promote elongation, but not nucleation, of microtubule assembly. To investigate this possibility, we added preformed microtubule seeds (see Materials and Methods) to tubulin (0.5 mg/ml) and \([\text{H}]\text{GTP} \) in the presence or absence of the peptides. Without any peptide additions, only a minimal increase in guanine nucleotide incorporation was observed; however, upon addition of peptide \( m_2 \), significant assembly was again observed. By contrast, peptides \( m_1 \) and \( m_3 \) failed to cause any significant increase of labeled guanine nucleotide incorporation into microtubules beyond background levels (Fig. 5). Thus, \( m_2 \) is the only peptide that can stimulate nucleation and elongation.

Discusssion

The experiments described in this report were designed to gain further insight about the microtubule-binding fragment of MAP-2. We have identified a common protease-accessible site in MAP-2 and tau proteins, and we have demonstrated that a single octadecapeptide corresponding to the second repeated sequence (from Val-1705 through Gly-1722 in murine MAP-2) promoted microtubule nucleation and elongation. Thus, a sequence amounting to \( \sim 1\% \) of the overall MAP-2 molecule is sufficient to interact with tubulin, but some additional considerations of MAP-2 structure seem appropriate.

There is now general agreement that initial proteolytic cleavage of MAP-2 yields two fragments (Vallee, 1980; Flynn et al., 1987). With thrombin, these initial cleavage products corresponding to values of 240,000- and 28,000-M, based on electrophoresis, are quite stable with regard to further degradation. A striking common structural feature in MAP-2 and tau emerges from the combined findings of Aizawa et al. (1988) and our studies. The former found that chymotryptic cleavage of the bovine tau proteins yielded a microtubule-binding fragment with the NH\(_2\) terminal sequence shown in Fig. 2, and we have now demonstrated that thrombin attacks a similarly accessible region in bovine MAP-2 (See also Fig. 2). The reader should note that both of these cytomatrix proteins have four proline residues in exact registration, and with the exception of the occurrence of a val-pro in the MAP-2 sequence, each of the prolines in both cleavage sites is preceded by a hydroxy-amino acid. Efforts to survey other known sequences in the GenBank database have indicated the uniqueness of these protease-accessible regions in tau and MAP-2; however, Earnshaw et al. (1987) described a centromere-binding position containing three prolines in exactly corresponding positions with little other structural relatedness to tau and MAP-2. The circular dichroism spectral data of Hernandez et al. (1986) indicates that uncleaved MAP-2 contains little, if any, alpha helical or pleated-sheet secondary structure; yet, the preferential action of the endoprotease thrombin at a single site suggests that MAP-2 may display
some "hinge-point" behavior akin to the protease-accessible region of myosin. This region may permit the projection arm to swing away from the microtubule surface. Certainly, the observed sedimentation coefficient of 4.5 (Hernandez et al., 1986) also suggests that MAP-2 has extended a flexible structure. The roughly spherical hemoglobin molecule, itself only one-third the molecular mass of MAP-2, has an almost identical sedimentation coefficient (Sanders et al., 1981). Chymotryptic cleavage between Tyr-128 and Ser-129 in the tau proteins may reflect a corresponding protease-accessible site of structural discontinuity between microtubule-binding and projection domains.

That the second repeated sequence in MAP-2 can promote microtubule assembly is indeed interesting. Lewis et al. (1988) had studied microtubule binding of an in vitro translation product spanning amino acids 1621-1722 (including the first and second repeated sequences). While they did not demonstrate promotion of microtubule assembly, Lewis et al. (1988) succeeded in showing that their radioactively labeled 100-residue polypeptide copurified through two cycles of assembly/disassembly with MAP-containing microtubule protein. We found that a single octadecapeptide is effective, even in the absence of MAPs. While we cannot discount the possibility of multiple binding of peptide m2 to tubulin, establishing the stoichiometry of ligand binding to a protein can be particularly challenging for ligands that bind in the millimolar concentration range. We should also emphasize that ~0.5 mM of the peptide m3 is needed to promote assembly, but MAP-2 is effective in the 1-5-µM range. It takes an even higher concentration (1.0 mM) of m2 peptide to displace intact MAP-2 from microtubules; yet a peptide containing more of the adjacent sequences of m2 can displace MAP-2 at lower concentrations (Joly, J., and D. Purich, unpublished observations). This may indicate that the several repeats in MAP-2 reinforce each other in promoting assembly or that our octadecapeptide cannot readily assume the assembly-promoting conformation. Obviously, the conformational latitude of the second repeated sequence could be greatly restricted by the other residues in the entire microtubule-binding domain of MAP-2. At this point, however, the combined findings of Lewis et al. (1988) and our observations attest to the importance of the second octadecapeptide in MAP-2 interactions with tubulin.

The peptide corresponding to the second repeated sequence has an overall isoelectric point that is more basic than the first and third octadecapeptides (e.g., m2, m3, and m4 have calculated isoelectric points of 10.5, 11.6, and 8.0). In the assembly buffer (pH 6.8), m3, m2, and m1 have overall charges of +3, +4, and +0.4, respectively. There are other indications that ionic interactions are important in MAP-2 binding to tubulin and/or microtubules. Flynn et al. (1987), for example, showed that the 28,000-Mr tubule-binding fragment of MAP-2 had an isoelectric point of ~10.5 whereas the larger projection-arm fragment was considerably more anionic (isoelectric point ~4.8). Moreover, anions such as polyglutamate and heparin sulfate are generally known to block MAP binding to microtubules, and the MAP binding site on tubulin is thought to involve the glutamate-rich carboxyl-termini of the tubulin heterodimer. Nonetheless, we cannot conclude that ionic interactions alone determine the effectiveness of peptides in promoting assembly.

Most recently, oligopeptide sequences from tau and a 190-kD bovine adrenal gland MAP were shown to possess the ability to polymerize microtubules (Ennulat et al., 1989; Aizawa et al., 1989). The first and second octadecapeptide repeats in tau were shown to promote microtubule polymerization, but the third was ineffective. The first repeated sequence in MAP-2 cannot induce microtubule assembly, unlike its counterpart in tau. The only significant difference between the two sequences is a lysine in place of a glycine near the carboxy terminus. This structural feature is an excellent candidate for a β-turn, and its disruption may preclude the ability of this peptide to stimulate microtubules. When this lysine is replaced by a glycine residue, the peptide can displace MAP-2 just as effectively as the m2 peptide (Joly, J., and D. Purich, unpublished observations). The assembly-promoting sequence in the 190-kD adrenal MAP was very similar to the first repeated sequence in tau and produced microtubules of normal morphology. We have compared the sequences (see Fig. 6) to identify common features in the assembly-promoting sequences. For this purpose, these peptide sequences were brought into registration with

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\begin{align*}
\text{m}_1 & \quad \text{val-lys-ser-lys-ile-gly-ser-thr-asn-ile-lys-tyr-glu-pro-lys-gly} \\
\text{t}_1 & \quad \text{val-arg-ser-lys-ile-gly-ser-thr-glu-asp-lys-his-glu-pro-lys-gly} \\
\text{ag} & \quad \text{lys-asn-val-arg-ser-lys-val-glu} \\
\text{m}_2 & \quad \text{val-thr-ser-lys-cys-gly-ser-leu-lys-arg-asn-ile-gly} \\
\text{t}_2 & \quad \text{val-thr-ser-lys-cys-gly-ser-leu-gly-asn-ile-lys-his-lys-pro-gly-gly} \\
\text{m}_3 & \quad \text{ala-gln-ala-val-glu} \\
\text{t}_3 & \quad \text{val-gln-ser-lys-glu-val-arg} \\
\end{align*}
\]

Figure 6. The octadecapeptide repeats of MAP-2 (m1, m2, and m3), and tau (t1, t2, and t3), and a corresponding sequence of the 190-kD adrenal gland MAP (ag) are shown. The (+) signs represent full positive charges at pH 6.8, whereas the delta represents partially positive imidazolium side-chain groups of histidyl residues. Plus and minus signs at the right of each peptide indicate assembly and no assembly, respectively.
respect to the pro-X-gly-gly regions of each. We note that all peptides have a common lysyl residue near their amino termini. Likewise, all assembly-promoting peptides contain one, or more, full positive charge(s) on residues near the pro-X-gly-gly sequence. Peptides m₁ and m₂ lack this latter feature and cannot stimulate assembly, suggesting that the partially charged histidyl residues offer insufficient ionic character at these sites. Interestingly, the presence of positive charge in the middle of the m₂ sequence does not preclude assembly; however, introduction of positive charge in the pro-lys-gly-gly of m₃ abolishes promotion of tubulin polymerization.

Finally, the observations that several octadecapeptides can promote assembly suggests a route for preparing low molecular mass modulators of microtubule assembly. As more information on the binding of MAPs to tubulin is developed, we may be able to improve the binding efficiency of these oligopeptides. Moreover, the availability of these peptides should permit additional studies of MAPs binding to other cytoskeletal elements including the neurofilament proteins.

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