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Peptides Corresponding to the Second Repeated Sequence in MAP-2 Inhibit Binding of Microtubule-Associated Proteins to Microtubules

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ABSTRACT: Bovine brain high molecular weight microtubule-associated proteins (MAPs) can be displaced from assembled tubules by peptides corresponding to the second of three nonidentical repeated sequences in mouse MAP-2. The octadecapeptide m_2 (VTSKCGSLKNIRHRPGGG) can release MAP-1b from MAP-containing microtubules, and the extended second-sequence peptide m_2' (VTSKCGSLKNIRHRPGGGRVK) displaces MAP-1a and MAP-1b as well as MAP-2a and MAP-2b. Peptides m_2 and m_2' stimulate tubulin polymerization in the absence of MAPs or microtubule-stabilizing agents, and m_2' acts as a competitive inhibitor of radiolabeled MAP-2 binding. The dissociation constant for MAP-2 binding to taxol-stabilized tubules was 3.4 μM in the absence of m_2' and 14 μM in the presence of 1.5 mM of the m_2' peptide. We estimate that the inhibition constant for peptide m_2' is about 0.5 mM, about 100 times lower than for the K_m of MAP-2. These observations suggest that the second repeated sequence in MAP-2 may represent an important recognition site for MAP binding to microtubules and that other structural features within MAP-2 may reinforce the strength of MAP-microtubule interactions.

Microtubule-associated proteins (MAPs) exhibit one of several properties: the ability to copolymerize with tubulin during microtubule assembly, the capacity to utilize tubulin or another MAP as substrates for enzyme-catalyzed modification, or the use of microtubules as the architectural framework for motility (Olmsted, 1986; Purich & Kristofferson, 1984). The first property is shared by the high molecular weight proteins (MAP-1 and MAP-2) as well as the τ proteins, and these proteins remain associated with reassembled microtubules during the course of microtubule protein purification. Recently, the cDNA-derived amino acid sequences of the murine MAP-2 (Lewis et al., 1988) and the murine τ (Lee et al., 1988) proteins have been defined, and these proteins were both found to contain a related triad of imperfectly repeated octadecapeptide sequences in their tubule binding regions. Oligopeptide analogues of the repeated sequences in murine τ and a 190-kDa bovine adrenal gland MAP can promote microtubule assembly (Ennulat et al., 1989; Aizawa et al., 1989). Likewise, we have demonstrated that analogues of the second repeated sequence of murine MAP-2 can promote microtubule self-assembly (Joly et al., 1989).

While several peptides corresponding to sequences in fibrous MAPs can stimulate microtubule assembly, very little is known about whether these synthetic peptides constitute the entire site necessary for MAP binding to microtubules. If the repeated sequences are indeed the primary sites of interaction, then those promoting tubule assembly in the absence of MAPs may also displace MAPs from microtubules or block their binding to microtubules. Moreover, we were motivated to learn whether a particular peptide and MAP display competitive

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binding behavior. We also wanted to compare the effectiveness of all three MAP-2 repeated peptide analogues in terms of MAP displacement from microtubules. Our experiments show that peptides corresponding to the second repeated sequence can displace MAP-1 and MAP-2 from recycled microtubule protein.

Materials and Methods

Reagents. [γ-^{32}P]ATP, ultrapure sucrose, and ammonium sulfate were purchased from ICN. Ultrapure area was purchased from Bethesda Research Laboratories, and Bio-Gel A 1.5M was from Bio-Rad. The catalytic subunit of cAMP-dependent protein kinase, ATP, bovine serum albumin, dithiothreitol, Pipes, Mes, EGTA, and GTP were from Sigma. Liquid scintillation cocktail 3a70 was obtained from Research Products International. [^{3}H]GTP was from Amersham and had a specific activity of 7 Ci/mmol. Taxol was a gift supplied by Dr. Matthew Suffness at the National Cancer Institute, Bethesda, MD.

Preparation of Proteins. Isotonic bovine brain microtubule protein was isolated according to the method of Karr et al. (1979) and stored at −80 °C after two cycles of assembly–disassembly. Hypotonic bovine brain microtubule protein was isolated by the method of Shelanski et al. (1973). Tubulin was prepared according to the method of Kristofferson et al. (1986). ^{32}P-MAP-2 was purified by the procedure of Herzog and Weber (1978) as modified by Flynn et al. (1987) except the purified protein was concentrated by ammonium sulfate precipitation after gel filtration chromatography. Unlabeled MAP-2 was prepared identically except the phosphorylation reaction was omitted prior to the gel filtration column. Synthetic peptides were prepared by Dr. Jan Pohl of the Microchemical Sequencing Facility at Emory University as described in Joly et al. (1989).

Isotonic Microtubule Experiments. Before use, the protein was carried through a third cycle of assembly–disassembly and the concentration of protein determined by the method of Bradford (1976). Synthetic peptides were weighed out just prior to use and dissolved in PEM buffer (100 mM Pipes, pH 6.8, 1 mM EGTA, and 1 mM $MgSO_4$) containing 1 mM dithiothreitol. Peptides were added at the indicated concentrations to 0.8 mg/mL isotonic microtubule protein with 0.5 mM GTP and 1 mM dithiothreitol and incubated at 37 °C for 20 min. The microtubules were subsequently stabilized with 10 μM taxol for 10 min at 37 °C. The samples (250 μL) were then centrifuged for 8 min at 300000g, 37 °C, in a Beckman TL 100.2 rotor. The pellets were dissolved in 8 M urea and analyzed by gel electrophoresis.

Competition with Radiolabeled MAP-2. All radiolabeled MAP-2 experiments were performed with polypropionate airfuge tubes which were coated with 10 mg/mL bovine serum albumin for 5 min and rinsed with PEM buffer just prior to use. This treatment reduces nonspecific binding of proteins to the walls of the centrifuge tubes. The radiolabeled MAP-2 was clarified prior to use for 20 min at 130000g in a Beckman airfuge to remove any aggregated or denatured protein. Phosphocellulose-purified tubulin was incubated at 5 mg/mL, 37 °C, with 1 mM GTP for 20 min and subsequently stabilized with 50 μM taxol for an additional 10 min. The microtubules were then diluted 20-fold into a solution containing 3 μM radiolabeled MAP-2 with either unlabeled MAP-2 or synthetic peptides for 20 min at 37 °C. The solution also contained 10 μM taxol and 1 mM GTP to maintain microtubule stability. The samples (100 μL) were then carefully loaded into coated airfuge tubes with the aid of a microcapillary pipetter onto a 50-μL layer of 20% (w/v) sucrose in PEM buffer warmed to 37 °C. The samples were centrifuged for 30 min at 130000g and the supernatants removed. The pellets were washed with 100 μL of 10 mg/mL bovine serum albumin in phosphate-buffered saline, pH 7.3, containing 0.1% Triton X-100 and resuspended in 100 μL of 8 M urea. Aliquots of 25 μL were taken for liquid scintillation counting.

Tritiated GTP Incorporation Assay. To determine the extent of microtubule assembly induced by synthetic peptides of MAP-2, the assay of Maccioni and Seeds (1978) as modified by Wilson et al. (1982) was used except the specific activity of the GTP was 7 μCi/mL instead of 20 μCi/mL. The conditions are identical with the assay in Joly et al. (1989).

Miscellaneous. Protein concentration determinations were performed according to Bradford (1976). Polyacrylamide gel electrophoresis was done by the method of Bloom et al. (1985) omitting sodium dodecyl sulfate in the separating and stacking gels and adding 2 M urea to the separating gel. Gels were stained with Coomassie Brilliant Blue R-250 and scanned with an LKB ultrascan densitometer.

Results

Oligopeptides corresponding to the repeated sequences in several MAPs promote tubulin polymerization, but we focused our attention on the action of synthetic peptides of the repeated sequences of MAP-2. These are designated as peptides m_1 (sequence = VKSKIGSTDNIKYQPKGG), m_2 (sequence = VTSKCGSLKNIRHRPGGG), and m_3 (sequence = AQAKVGSLDNAHHVPGGG), corresponding to positions 1674–1691, 1705–1722, and 1737–1754, respectively, of the murine MAP-2 sequence (Lewis et al., 1988).

Displacement of MAPs from Recycled Microtubule Protein by MAP-2 Repeated Sequence Peptides. While only peptide m_2 promotes microtubule self-assembly, we were interested in determining whether m_1 and m_3 might also bind to assembled tubules and displace MAP-2. We therefore assessed the ability of the MAP-2 repeated sequence peptides to displace HMW MAPs from assembled microtubules. For this purpose, we used microtubule protein isolated by the isotonic extraction method (Karr et al., 1979) because such protein is rich in both MAP-1 and MAP-2. As shown in Figure 1A, SDS gel electrophoretograms of the microtubule fractions, after assembly and centrifugation, indicate that MAP-2 was only susceptible to displacement by a 21 amino acid peptide, m_2', corresponding to the m_2 sequence above plus residues RVK at the C-terminus. This extended peptide was synthesized because two of the three additional residues are basic amino acids thought to be important to interact with the anionic carboxyl termini of α- and β-tubulin. These residues are similar to the C-terminus of an assembly-promoting peptide of the 190-kDa adrenal gland MAP which contains RAK at its end. Interestingly, MAP-1b as selectively displaced by peptide m_2 and all high molecular weight MAPs were removed from microtubules in the presence of peptide m_2'. Densitometry tracings of lanes 2, 5, and 6 indicate the profiles of MAPs with m_2, m_2', and no peptide, respectively (Figure 1B). MAP-1b may be preferentially displaced before other high molecular weight MAPs due to its lower affinity for in vitro microtubules as previously noted by Bloom et al. (1985) and Noble et al. (1989). Furthermore, SDS gel electrophoresis confirmed that there was reciprocal recovery of MAPs in the supernatant fraction after release from polymerized tubules upon incubation with peptides corresponding to the second repeated sequence (data not shown).

Because the 21 amino acid peptide was more effective in displacing MAPs, we wished to compare it with the m_2 peptide with regard to the promotion of microtubule assembly (see

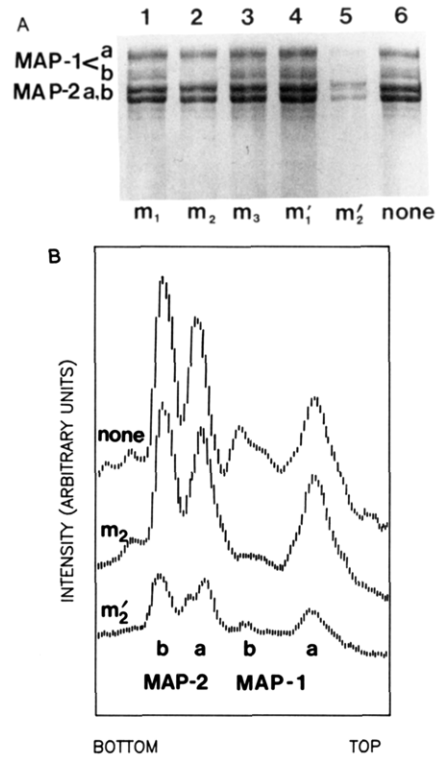

FIGURE 1: (A) Effects of MAP-2 peptides on MAP binding to microtubules. Coomassie Blue staining of proteins in pelleted microtubule fractions after electrophoreses on a 4% polyacrylamide gel: lane 1, m_1; lane 2, m_2; lane 3, m_3; lane 4, m_1'; lane 5, m_2'; lane 6, no peptide. All peptides were added to a concentration of 2.0 mM. (B) Densitometry of the Coomassie Blue stained gel. The upper trace represents lane 6, the middle trace is lane 2, and the lower trace is lane 5.

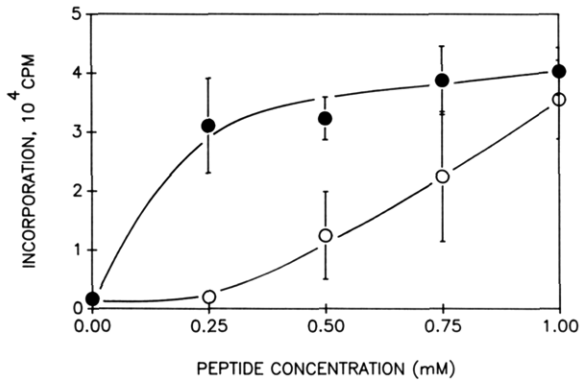

FIGURE 2: Comparison of the stimulation of tubulin polymerization by peptides m_2 and m_2'. The incorporation of tritiated GTP into microtubule polymer was measured to examine the assembly-promoting activity of m_2 and the extended analogue m_2'. Phosphocellulose-purified tubulin was used at 1 mg/mL. The open circles represent m_2 and the closed circles m_2'. Error bars represent standard deviations from three replicate points.

Figure 2). We found that tubulin polymerization was considerably more effective in the presence of m_2'. Together, these observations suggest that only peptides corresponding to the second repeated sequence can displace MAPs from assembled microtubules. The data also indicate that both MAP-1 and MAP-2 can be displaced by peptide m_2', suggesting further that this peptide may bind to common, or closely overlapping, sites on microtubules. Peptides m_1, m_2, and m_3 were otherwise without effect, as was an analogue of m_1, m_1', containing a Gly in place of Lys toward the C-terminus. This substitution near the carboxyl terminus of the peptide removed a positive charge at physiological pH but conserved a possible β-turn structure present in all murine and bovine τ repeats and in

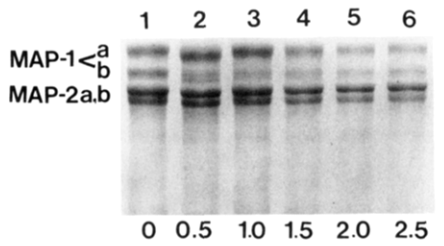

FIGURE 3: Effects of increasing the m_2' concentration on high molecular weight MAP binding to microtubules. Peptide m_2' was added to isotonic microtubule protein to the final millimolar concentration indicated at the bottom of each lane. After centrifugation, the pellet fractions were analyzed by gel electrophoresis. The Coomassie Blue staining of a 4% polyacrylamide gel is shown.

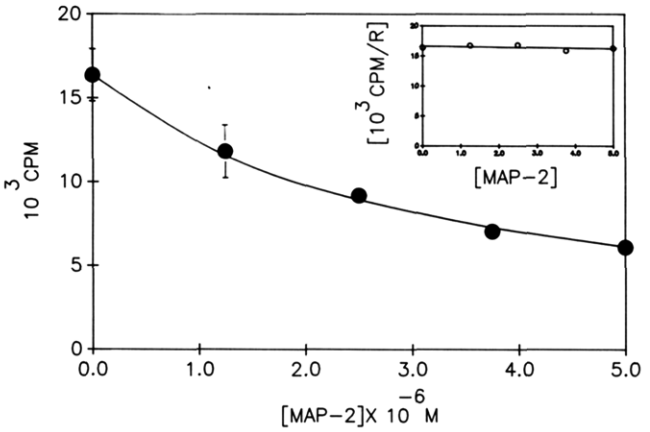

FIGURE 4: Displacement of trace phosphorylated MAP-2 from taxol-stabilized microtubules by unlabeled MAP-2. See Competition with Radiolabeled MAP-2 under Materials and Methods. The molarity of MAP-2 was calculated by using a molecular weight of 200000.

the two other murine MAP-2 repeats (Lewis et al., 1988; Lee et al., 1988; Himmler et al., 1989).

The results shown in Figure 3 demonstrate that the extended second repeated sequence peptide m_2' removed MAP-1 and MAP-2 from microtubules in a concentration-dependent manner. Again, we used densitometry to gauge the extent of MAP depletion in the assembled tubule fraction, and we found that the concentration of peptide m_2' that displaces 50% of MAP-2 is about 1.5–2.0 mM (data not shown). Thus, the level of peptide m_2' is about 4 times the concentration needed to promote tubulin polymerization in the absence of MAPs or microtubule-stabilizing agents (see Figure 2).

Radiolabeled MAP-2 Binding to Microtubules. While the findings presented in Figures 1 and 3 provide clear evidence of MAP displacement, we were interested in developing a quantitative displacement/binding assay. We incubated MAP-2 with cAMP-stimulated protein kinase and [γ-^{32}P]ATP under conditions that we have found to result in the incorporation of about 1–1.5 phosphoryl groups per MAP-2 molecule (Flynn et al., 1987). Because extensive MAP-2 phosphorylation can alter the affinity of MAP-2 to microtubules (Murthy & Flavin, 1983; Hoshi et al., 1988), we wanted to compare the binding behavior of our trace phosphorylated MAP-2 with unmodified MAP-2 isolated by the standard recycling preparation protocol (Shelanski et al., 1973; Herzog & Weber, 1978). Accordingly, we incubated taxol-stabilized microtubules with the phosphorylated MAP-2 in the presence of several concentrations of unphosphorylated MAP-2 (Figure 4). The data points show that the amount of radiolabeled MAP-2 bound to microtubules decreases at increasing concentrations to the unlabeled MAP-2; the solid line is the theoretical curve calculated on the basis of isotopic dilution,

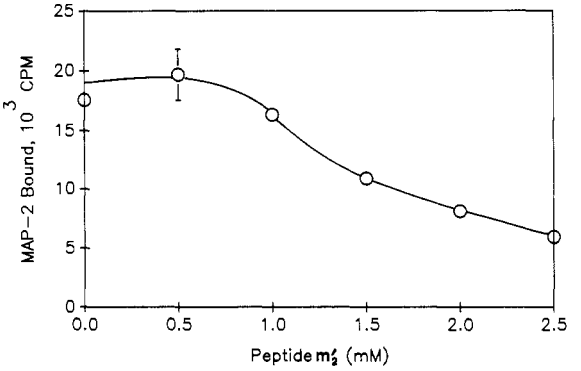

FIGURE 5: Displacement of trace phosphorylated MAP-2 from taxol-stabilized microtubules by peptide m_2'. See Competition with Radiolabeled MAP-2 under Materials and Methods. Error bars represent standard deviations from three replicate points.

using the ratio $R = [\text{MAP-2*}]/([\text{MAP-2}] + [\text{MAP-2*}])$, where labeled protein and unlabeled protein are MAP-2* and MAP-2, respectively. The data normalized with respect to R (Figure 4 inset) indicate that the relative affinities of both MAP-2 species are the same within experimental error.

Displacement of Labeled MAP-2 by a Second Repeated Sequence Peptide. To gain a more quantitative view of MAP-2 displacement, we next examined the amount of microtubule-bound [^{32}P]MAP-2 as a function of the concentration of peptide m_2'. As shown in Figure 5, MAP-2 is displaced by this peptide, but the desorption process is not described by a typical hyperbolic dissociation curve. The basis of the slight stimulation of MAP-2 binding at 0.5 mM peptide m_2' is unclear, but careful inspection of the MAP-2 band in lane 2 of Figure 3 revealed a similar behavior. Thus, we find that the same displacement behavior is observed whether or not MAP-2 is trace-phosphorylated by the cAMP-stimulated protein kinase.

Competitive Binding of Peptide m_2' and MAP-2. We were particularly interested in the mode of inhibition of MAP-2 binding to microtubules by the extended second repeated sequence peptide m_2'. We therefore conducted a series of radiolabeled MAP-2 binding measurements over the concentration range of MAP-2 shown in Figure 6. These experiments were carried out in the absence or presence of 1.5 mM peptide m_2'. Our results indicate that peptide m_2' does indeed act as a competitive inhibitor of MAP-2 binding. The data presented here indicate that the MAP-2 interaction with microtubules is defined by a single class of binding sites. The maximal extent of MAP-2 binding was found to correspond to one molecule of MAP-2 per four molecules of polymerized tubulin dimers. The dissociation constant for MAP-2 binding to microtubules was 3.4 μM in the absence of peptide m_2' and 14 μM in the presence of a 1.5 mM sample of this peptide. From the change in slope, we estimate that the m_2' inhibition constant was about 0.5 mM. Thus, we estimate that the dissociation constant for MAP-2 is about 100 times less than the corresponding constant for peptide m_2.

Discussion

Earlier findings suggested that MAP-2 interacts with microtubules largely through ionic forces. Aside from the ability of intermediate salt concentrations (e.g., 0.4–0.6 M NaCl) to block MAP binding to tubules, the inhibitory action of polyanions and polycations should also be noted. Among the polyanionic inhibitors of assembly are RNA and polyglutamate (Bryan, 1976; Bryan et al., 1975), phosphatidylinositol (Yamauchi & Purich, 1987), and estramustine phosphate (Wallin et al., 1985). These agents are thought to bind to the microtubule binding region of MAP-2, and Flynn et al. (1987) demonstrated that the M_r 28 000 microtubule binding fragment of MAP-2 has an isoelectric point of 10.0–10.5. Polycations also bind to microtubules and promote assembly of tubulin (Erickson & Voter, 1976; Erickson & Scott, 1977). MAP binding is thought to occur at the glutamate-rich C-termini of the tubulin α and β chains, and the polycations presumably block these MAP binding sites on tubulin and/or microtubules. Indeed, subtilisin treatment of tubulin results in the loss of small glutamate-rich C-terminal sequences from both α- and β-tubulin (Paschal et al., 1989). Tubulin proteolyzed in this manner readily assembles but fails to bind MAPs (Serrano et al., 1984).

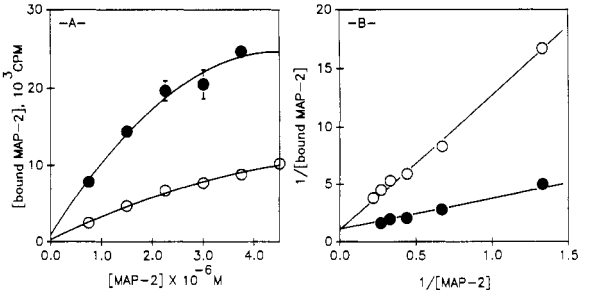

FIGURE 6: Radiolabeled MAP-2 binding to taxol-stabilized microtubules in the presence and absence of 1.5 mM m_2' peptide. (Panel A) Plot of bound MAP-2 versus total MAP-2 in the absence (closed circles) and presence (open circles) of m_2'. In this experiment, the microtubules were diluted into a solution containing radiolabeled MAP-2 with or without 1.5 mM peptide. (Panel B) Double reciprocal plot of MAP-2 binding from panel A: with peptide (open circles); without peptide (closed circles). Error bars represent standard deviations from three replicate points.

MAP-1 and MAP-2 are both displaced from microtubules in the presence of peptides based on the second repeated sequence. This finding suggests that peptides m_2 and m_2' can bind at or near the site(s) of MAP-1 interaction with microtubules in a way that interferes with MAP-1 binding. Until we can also develop a quantitative binding assay for MAP-1, we cannot discern whether these peptides act as competitive inhibitors, as was found to be the case for MAP-2. At the same time, we can be confident that peptides m_2 and m_2' will be found to competitively inhibit MAP-2c binding to microtubules. This assumption is based on the interesting finding of Papandrikopoulo et al. (1989) that the embryonic MAP-2c has an identical amino acid sequence in the C-terminal microtubule binding motif as the adult forms, MAP-2a and MAP-2b. The embryonic protein lacks 1342 amino acid residues spanning positions 147–1519 of adult rat MAP-2 as a result of an alternative splicing event.

It is noteworthy that a synthetic peptide derived from the 190-kDa bovine adrenal gland MAP efficiently displaced the protein at a concentration of 600 μM (Aizawa et al., 1989). However, the apparent dissociation constant of the 190-kDa MAP for microtubules is 180 μM while the dissociation constant of MAP-2 is 1–5 μM. Therefore, the finding that a high level of MAP-2 synthetic peptide is required to displace MAP-2 is not surprising.

The finding that 4 times the level of peptide is required to displace MAPs than is necessary for tubulin polymerization may indicate that not all the available MAP binding sites of tubulin are occupied during nucleation and elongation of microtubules. Of course, for any competitive binding process, the apparent dissociation constant of one ligand is affected by the concentration and dissociation constant of the competitive ligand. Thus, the apparent constant K_a' equals $K_a(1 + [B]/K_b)$, and the other apparent constant, K_b', likewise equals

$K_b(1 + [A]/K_a)$. As a result, the assembly promotion assay is carried out with the peptide analogue in the absence of MAP-2, and the dissociation constant will necessarily be lower. The higher level of peptide required in the displacement assay may be needed to completely block the MAP binding sites on these taxol-stabilized microtubules.

That peptides corresponding to the second repeated sequence of MAP-2 can both promote assembly and displace MAPs opens the way for developing high-affinity peptide analogues. We now have functional assays for the assembly-promoting and MAP-displacing characteristics of such peptides, and the latter can be extended by the electrophoresis experiments to test for the specificity of MAP displacement (i.e., preferential desorption of a particular MAP). We have already found that the extended second sequence peptide m_2' is more effective than peptide m_2. Part of such an effort will involve the identification of those features in peptides m_1 and m_3, that preclude promotion of tubulin polymerization or blocking of MAP binding. We had thought that the failure of peptide m_1 to promote assembly resulted from a lysyl residue toward the carboxyl terminus. Nonetheless, we find that an m_1 peptide analogue with PKGG converted to PGGG does not displace MAP-2 as we mistakenly reported earlier.

At present, the affinity of the synthetic peptides toward microtubules is low. Although arguments can be made for the necessity of flanking sequences or multiple repeats, the affinity is 100-fold lower than that of MAP-2, and also lower by a factor of 5 than the effective concentration of τ peptides. However, the addition of the next three amino acid residues in the murine MAP-2 sequence yields a more effective stimulator of tubulin polymerization compared to m_2 and approximately equal to the τ peptides (Ennulat et al., 1989). In this regard, it would be interesting to know the association constants for various polycations in comparison with their microtubule assembly and MAP competition effects, and whether MAP displacement by polycations is due to competitive inhibition, since this bears directly on the conclusions made concerning the specificity of the m_2 effects.

Finally, there is mounting evidence that a group of microtubule-associated proteins achieve binding to microtubules by way of multiple nonidentical repeated octadecapeptide sequences (Lewis et al., 1988; Himmler et al., 1989). The stimulation of tubulin polymerization by synthetic peptides corresponding to several repeated sequences is consistent with, but does not prove, this hypothesis. On the other hand, the findings presented here indicate that peptides resembling the second repeated sequence of MAP-2 can displace MAP-2 from microtubules and can competitively inhibit MAP-2 binding. Because only these peptides promote tubulin polymerization, we are drawn to the conclusion that these peptides do indeed mimic MAP binding to microtubules.

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