Exploring the Microtubule-Binding Region of Bovine Microtubule-Associated Protein-2 (MAP-2): cDNA Sequencing, Bacterial Expression, and Site-Directed Mutagenesis†

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ABSTRACT: A 1.1 kilobase fragment of bovine microtubule-associated protein-2 (MAP-2) cDNA coding for bovine MAP-2 microtubule-binding region (*MTBR*) was sequenced. Relative to mouse, rat, and human MAP-2, we observed striking preservation of primary structure, even beyond the sequence and spacing of the three nonidentical peptide repeats responsible for microtubule-binding interactions. For further analysis of microtubule−MAP interactions using site-directed mutagenesis, we developed a bacterial expression system coding for the MT-binding fragment of MAP-2 starting at the thrombin cleavage site (position 1629) and continuing to the C-terminus. This MT-binding fragment was purified to homogeneity by taking advantage of the unusual heat-stability and isoelectric properties of this cytomatrix component. We found that the MT-binding domain readily promoted tubulin polymerization, and the critical tubulin concentration was reduced in the presence of this recombinant protein. Because a second repeated sequence analogue can promote tubulin polymerization as well as displace the MT-binding region of MAP-2, this study was designed to learn more about the importance of each repeated sequence in MT binding. Accordingly, we mutated the first and third sequences to resemble the second repeated sequence, thereby generating the mutants designated m_{12}-m_2-m_3, m_1-m_{32}, and m_{12}-m_2-m_{32}. These recombinant proteins bound with an affinity comparable to or slightly better than equal concentrations of wild-type MT-binding fragment. Likewise, when the first or third sequence was replaced by an exact copy of the second octadecapeptide repeat, there was little, if any, increase in binding affinity, as reflected in the ability of mutant MT-binding fragments to promote tubulin polymerization. We can thus conclude that the binding energies associated with each of the three second-sequence repeats were not additive, and these findings fortify the conclusion that the second sequence repeat plays a dominant role in microtubule binding.

Microtubule-associated protein-2, a cytomatrix component of neuronal dendritic processes, interacts with microtubules (MTs)[1] and is thought to stabilize the neuronal cytoskeleton by forming cross-links between microtubules (Olmsted, 1986). A single MAP-2 gene appears to serve as a precursor for several different protein species that arise from alternative splicing of a common MAP-2 mRNA precursor. MAP-2ab are high-molecular-weight forms appearing in dendrites of adult neurons (Papandrikopoulou *et al.*, 1989), and MAP-2c is a low-molecular-weight counterpart containing the same extreme amino and carboxy termini found in the adult forms. MAP-2ab can be readily cleaved by serine proteases (Vallee & Borisy, 1977) into on N-terminal projection-arm domain and a C-terminal microtubule-binding region, the latter designated here as MAP-2 *MTBR*. Unlike chymotrypsin and trypsin, however, the arginine-specific protease thrombin produces stable limit digestion products after cleavage at the protease-accessible hinge region of MAP-2 (Flynn *et al.*, 1987). The MT-binding fragment is quite basic with an isoelectric point of about 10.3, and the projection-arm is acidic with a corresponding isoelectric point of approximately 4.8 (Flynn *et al.*, 1987). The 203-amino acid residue MT-binding fragment displays the same apparent affinity as MAP-2ab for microtubules (Joly *et al.*, 1989). This region includes a triad of regularly spaced nonidentical octadecapeptide repeats (Lewis *et al.*, 1988), and analogues corresponding to the second-repeated sequence promote tubulin polymerization (Joly *et al.*, 1989) and displace MAP-2 from assembled microtubules (Joly & Purich, 1990).

Most of the biochemical characterization of MAP-2 has been achieved through characterization of the heat-stable bovine or porcine protein, while the amino acid sequence was deduced using mouse or rat MAP-2 cDNA (Lewis *et al.*, 1988; Kindler *et al.*, 1990). MAP-2 phosphorylation is known to alter MT assembly properties (Jameson & Caplow, 1980), and recent studies have shown that phosphorylation enhances the dynamic instability of brain microtubules *in vitro* (Raffaelli *et al.*, 1992). Our interest in analyzing MAP interactions with microtubules through site-directed mutagenesis led us to isolate and sequence the corresponding 1.1 kilobase fragment of bovine brain MAP-2 cDNA. We also developed an efficient bacterial expression system and purification method to isolate this microtubule-binding region of MAP-2, which provide for further characterization of the

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[1] Abbreviations: MT, microtubule: MAP-2 *MTBR*, generic designation of the microtubule binding region; IPTG, isopropyl β-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PCR, polymerase chain reaction.

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microtubule-binding properties by using site-directed mutagenesis. This allowed us to obtain new insights regarding the interactions of the nonidentical peptide repeats with microtubules.

EXPERIMENTAL PROCEDURES

Materials

[α-^{32}P]dATP, SDS, and sucrose were ICN products. Amersham, Inc., supplied the [α-^{35}S]dATP and their "Oligonucleotide-Directed *in vitro* Mutagenesis System" (version 2.1). Reverse transcriptase was from Promega, and Klenow and T4 DNA ligases were from New England Biolabs. Restriction endonucleases were purchased from New England Biolabs or Promega. The bovine brain cDNA library was obtained from Clonetech, Inc., and the Sequenase (version 2.0)DNA sequencing kit was a U.S. Biochemicals product. Expression vector pETh-3b, a derivative of pET-3b described by Studier *et al.* (1990), was a gift from Dr. Donald R. McCarty of the Department of Vegetable Crops, University of Florida. Chloramphenicol and acetate kinase were purchased from Boehringer-Mannheim. Ampicillin, isopropyl β-thiogalactopyranoside (IPTG), 2-(*N*-morpholino)ethanesulfonic acid (MES), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), EGTA, $MgCl_2$, dithiothreitol, protease inhibitors, lysozyme, *Tris*-HCl, trifluoroacetic acid, and GTP were from Sigma. Phosphocellulose-P11 was a Whatman product. Taxol was obtained from Dr. Matthew Suffness of the National Cancer Institute, Bethesda, MD.

Methods

Cloning and Sequencing the Bovine MAP-2 MT-Binding Region. The mouse cDNA sequence data of Lewis *et al.* (1988) served as the basis for preparing polymerase chain reaction (PCR) primers spanning the amino acid sequences 5359−5388 (sense-primer) and 5643−5665 (antisense-primer). We employed standard molecular biological techniques (Sambrook *et al.*, 1989). Bovine brain RNA was isolated and transcribed into cDNA with reverse transcriptase using random hexamer primers. The polymerase chain reaction was then carried out for 30 cycles, products were fractionated on agarose gels, and bands were observed at sizes corresponding to 300 base pairs. These were electro-eluted and treated with mung-bean nuclease to remove single-stranded ends, then ligated into the *Sma*I site of M13mp19, and sequenced. A MAP-2 clone was identified and subsequently ratiolabeled for use as a probe in the screening of a 2λgt11 bovine brain cDNA library. Approximately 300 000 plaques were initially screened using duplicate filter lifts. Positives were picked and plated; two additional rounds were then performed, and two sets of clones were plaque-purified. One clone contained a 1.1 kilobase insert after a λ liquid lysate preparation and *Eco*RI digestion. This 1.1 kilobase fragment was introduced into pUC19 for subcloning and sequencing. The resulting plasmid, pJJ1, was grown in large quantity and digested with *Eco*RI, and the 1.1 kilobase insert was purified from an agarose gel. Several subclones were generated by digestion with *Hae*III and *Alu*I and subsequently sequenced. This approach allowed us to locate two unique internal restriction sites (Ba*m*HI and *Sph*I) from which three fragments spanning the entire 1.1 kilobase MT-binding region were generated. These fragments were subcloned into both M13mp18 and M13mp19 and sequenced in both orientations.

Vector Construction. Plasmid pJJ1 was digested with *Sac*I and *Xba*I which served to remove a fragment between a *Sac*I site in the 3′-untranslated region of the clone and an *Xba*I site in the pUC19 polylinker. A short synthetic double-stranded oligonucleotide with *Sac*I and *Xba*I ends was ligated into the vector to recreate a circular double-stranded plasmid lacking the *Eco*RI site at the 3′ end of the MAP-2 clone. The resulting plasmid was digested with *Eco*RI (at the 5′ end of the clone) and *Hinc*II (adjacent to the *Xba*I site in the pUC19 polyliner) to liberate the 1.1 kilobase MAP-2 clone. This was then ligated into the *Eco*RI and *Eco*RV sites in the polylinker of the expression vector pETh-3b (McCarty *et al.*, 1991) which had been previously digested with *Sph*I and *Pvu*II, the ends made blunt with T4 DNA polymerase, and ligated. Removal of this *Sph*I−*Pvu*II fragment destroyed the *Sph*I site and removed two *Ppu*MI sites so that these sites in the MAP-2 cDNA fragment could be subsequently employed. The resulting plasmid, pRC3, allows for the expression of the entire 323-amino acid C-terminal fragment of MAP-2 fused to the first 11 amino acids of the T7−gene 10 product. Plasmid pRC7 expressing the C-terminal 203-amino acid microtubule-binding domain, described by Joly *et al.* (1989), was prepared by digestion with *Ppu*MI (at the thrombin cleavage site in the MAP-2 amino acid sequence) and *Nhe*I (adjacent to the start codon), rendering the ends blunt with Klenow, followed by ligation. The resulting sequence was in-frame and coded for the 203-amino acid C-terminal MAP-2 fragment preceded by the sequence Met-Ala-Arg (the arginine is found in the native MAP-2 sequence).

Plasmids expressing the m_{12}-m_2-m_3, m_1-m_2-m_{32}, and m_{12}-m_2-m_{32} mutants were created using the Amersham kit for oligonucleotide-directed mutagenesis. A *Bam*HI−*Sph*I fragment from the MAP-2 cDNA (containing the m_2, m_3, and part of the m_1 repeated sequences) was ligated into the polylinker of M13mp19 so that a single-stranded DNA template could be produced. Synthetic oligonucleotides complementary to the m_1 and m_3 regions but containing the desired substitutions were used to carry out the mutagenesis. Before expression and purification of the mutant proteins, the resultant vectors were screened and sequenced to confirm that the desired mutants were made.

Bacterial Expression and Protein Purification. Expression plasmids were transformed into *Escherichia coli* BL21(DE3) and grown on LB plates containing 50 μg/mL ampicillin and 50 μg/mL chloramphenicol. A single colony was used to inoculate a 5 mL LB culture containing 50 μg/mL ampicillin and 50 μg/mL chloramphenicol and grown overnight at 37 °C. This culture was diluted into 500 mL of LB containing 50 μg/mL ampicillin and 50 μg/mL chloramphenicol and grown at 37 °C to an optical density of 0.4 (600 nm). IPTG was added at a concentration of 1 mM and growth continued for 2 h. Cells were harvested by centrifugation at 5000*g* for 5 min and resuspended in 20 mL of buffer (80 mM PIPES, 1 mM EGTA, and 1 mM $MgCl_2$, pH 6.8). They were pelleted again at 5000*g* for 5 min and resuspended in 4 mL of a buffer containing 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 40 μg/mL DNase, 40 μg/mL RNase, 100 μg/mL *N*-tosyl-L-phenylalanine chloromethyl ketone, 100 μg/mL N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, and 0.1 mM phenylmeth-

anesulfonyl fluoride. This suspension was subjected to two passes through a French pressure cell at 950 atm. Lysozyme was added to 1 mg/mL, and the solution was incubated at room temperature for 15 min with occasional stirring. This fluid was then layered onto 12 mL of 25% sucrose (w/v) containing 100 mM Tris-HCl (pH 8.0) and centrifuged at 14000*g* for 20 min at 20 °C. The resulting pellet containing insoluble inclusion bodies was rinsed with a 50 mM Tris-HCl buffer (pH 8.0) and resuspended using a Dounce homogenizer in 1 mL of 50 mM Tris-HCl (pH 8.0) buffer, containing 200 mM NaCl and 1 mM dithiothreitol. This suspension was then rapidly heated to a final temperature of 80 °C and held for 10 min to solubilize the heat-stable MAP-2 microtubule-binding fragment. Subsequent incubation on ice for 20 min and centrifugation at 400000*g* for 6 min served to remove most of the aggregated contaminating proteins. The supernatant predominantly contained intact MAP-2 MT-binding fragment, and this was passed through a 0.22 μm filter and subjected to reverse-phase chromatography using a Brownlee large-bore C_{18} reverse-phase column. A linear, two-step gradient from pure H_2O to 36% (v/v) acetonitrile in water after 45 min, and to 50% (v/v) acetonitrile in water after 75 min, caused the pure product to elute at approximately 52 min after the gradient commenced. [Trifluoroacetic acid (0.1% v/v) was present throughout the gradient.] Fractions containing pure MAP-2 microtubule-binding domain were pooled and dried in a Savant Speedvac concentrator, with subsequent resuspension in 1 mL of MEM buffer (100 mM MES, 1 mM EGTA, and 1 mM $MgCl_2$, pH 6.8). The solution was aliquoted, frozen in liquid nitrogen, and stored at −70 °C for later use.

Assembly Assays and Critical Concentration Measurements. Bovine brain microtubule protein and tubulin were prepared as outlined elsewhere (Joly & Purich, 1990). Tubulin polymerization experiments were carried out in PEM buffer (80 mM PIPES, 1 mM EGTA, and 1 mM $MgCl_2$, pH 6.8) with 1 mM GTP, and a GTP-regenerating system composed of 2 units/mL acetate kinase, and 10 mM acetyl phosphate. Tubulin (10 μM) was assembled with various concentrations of recombinant MT-binding fragment. The assembly process was monitored using a Beckman DU-2 spectrophotometer at 350 nm in a cuvette thermostated at 37 °C. Critical concentration determinations were made by assembling tubulin at 20 μM with 3.4 μM recombinant MT-binding fragment. After a plateau level of polymerization was reached, the apparent absorbance at 350 nm was recorded. Tubulin was diluted to 15, 10, and 5 μM in the presence of a constant level of MT-binding domain (3.4 μM). The absorbance was recorded at each dilution point only after steady-state polymerization was reached.

Competitive Binding Assays. Tubulin (50 μM) was permitted to self-assemble at 37 °C for 20 min followed by the addition of 50 μM taxol for an additional 10 min at 37 °C (Joly *et al.*, 1990). Microtubules were diluted to 2.5 μM in PEM buffer with 1.0 mM GTP, 10 μM taxol, 1.0 mM dithiothreitol, 10 μM recombinant MT-binding fragment, and the indicated amounts of competitor peptide. After 20 min incubation at 37 °C, samples were centrifuged in a Beckman Airfuge through a 20% (w/v) sucrose cushion at 100000*g* for 30 min at 37 °C. Pellets and supernatants were analyzed by electrophoresis according to the procedure of Laemmli (1970) and visualized after Coomassie staining. MAP-2 *MTBR* in the pellet fractions was determined by densitometry.

Protein was determined by the Bradford (1976) method with bovine serum albumin used as a standard. SDS gel electrophoresis was performed by the Laemmli (1970) method.

RESULTS

Bovine Brain MAP-2 cDNA Sequence and Deduced Amino Acid Sequence. To gain further insight concerning the subsite interactions of the three nonidentical repeated sequences and possibly other regions within the bovine brain MAP-2 microtubule-binding domain, we have utilized molecular cloning techniques to isolate a 1.1 kilobase region corresponding to the C-terminus of MAP-2. For this purpose, we screened a bovine brain cDNA library with a bovine probe obtained by PCR amplification of bovine brain cDNA using oligonucleotide primers based on the mouse brain MAP-2 cDNA sequence. This approach allowed us to avoid sequence artifacts arising from the PCR technique and to maximize hybridization by using the bovine probe with a bovine library. Because the cDNA library had an average insert size of 1−2 kilobases, and because the carboxy-terminal microtubule-binding region of MAP-2 only spans 600−700 base pairs, this strategy promised to provide us with an intact cDNA fragment corresponding to the entire MT-binding region. Indeed, as shown in Figure 1, dideoxy sequencing confirmed that this was the case, and our fragment contained the C-terminal-binding domain beginning at a position corresponding to amino acid 1509 in the murine sequence and extending past the stop codon into the 3′-untranslated region. We have previously demonstrated that a thrombin cleavage fragment of MAP-2 fully retains the tubule-binding properties of intact MAP-2, and we can now assign this sequence as that spanning the region from residues 1629 to 1828 of the mouse sequence. The exceptions are three additional amino acids (HTP) in the bovine sequence (1631−1633), an alanine-for-glycine substitution at 1639, and a glycine-for-serine change at position 1788. The additional HTP sequence near the thrombin cleavage site was also confirmed by Edman microsequencing (Joly *et al.*, 1989). Dingus *et al.* (1991) also carried out Edman analysis of a chymotrypsin cleavage fragment of bovine MAP-2, and their fragment can now be definitively localized at positions corresponding to amino acids 1526−1544. Most significantly, the three nonidentical repeated sequences which are boxed in Figure 1 are identical in bovine and murine sequences, attesting to the validity of earlier work using these sequences to investigate interactions of bovine brain MAP-2 with bovine brain microtubules (Joly *et al.*, 1989; Joly & Purich, 1990).

Bacterial Expression and Purification of the Bovine Brain MAP-2 MT-Binding Domain. To achieve efficient expression of the microtubule-binding domain of MAP-2 in *E. coli*, we utilized the pETh-3b expression vector. This plasmid has the advantage that protein expression is under the control of the T7 promoter, and expression is carried out in the *E. coli* vartiant BL21(DE3) which contains an integrated copy of the T7 RNA polymerase gene under control of the *lac*UV5 promoter (Studier *et al.*, 1990). Accordingly, expression commences upon the addition of IPTG to the bacterial culture medium. As shown in Figure 2, the MT-binding fragment of MAP-2, migrating with a molecular weight of 28 000, can be readily purified to homogeneity in three steps. Lane 1 is the cell-free extract, and lane 2 illustrates the state of

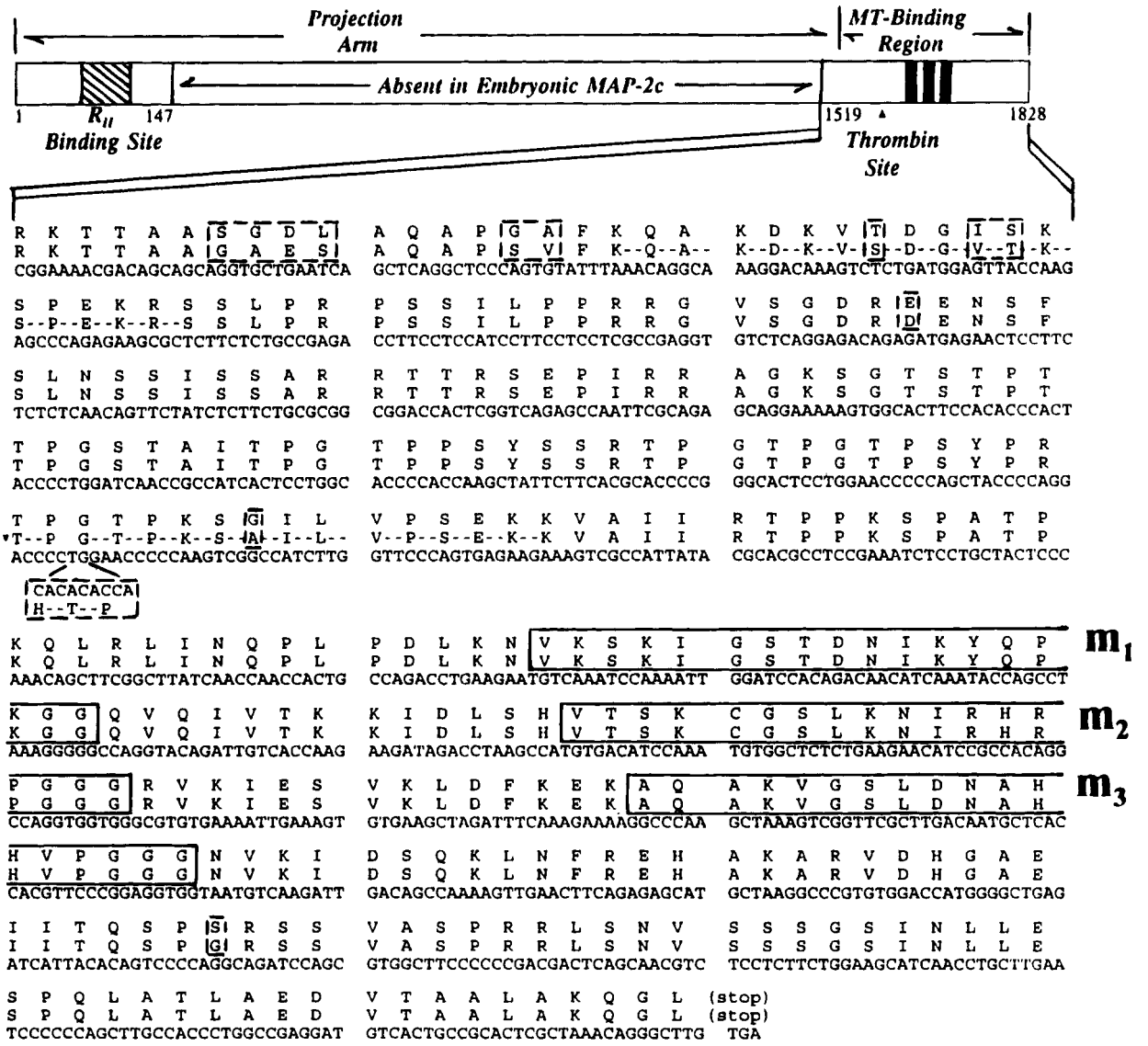

FIGURE 1: General structural organization of brain MAP-2, including nucleotide and deduced amino acid sequences of the bovine microtubule-binding region (*MTBR*). The schematic above includes the N-terminal cAMP protein kinase regulatory subunit-binding site, the C-terminal microtubule-binding region, and the three imperfectly repeated sequences. The latter are indicated in the schematic as vertical bars and in the sequence as boxed areas. The mouse amino acid sequence (top row) of the microtubule-binding region serves as a means to align the corresponding bovine MAP-2 sequence (middle row). Any exceptions to exact amino acid identity are indicated in dashed boxes. The corresponding bovine MAP-2 cDNA sequence is indicated on the bottom row. Confirming gas-phase amino acid sequence data for positions 1526−1544 and 1629−1645, obtained with chymotryptic (Dingus *et al.*, 1991) and thrombin (Joly *et al.*, 1989) cleavage products, are hyphenated in the sequence.

purification obtained upon centrifugation through a sucrose cushion to obtain presumptive protein inclusion bodies rich in the MT-binding fragment. Lane 3 shows the level of purity after heat-treatment at 80 °C for 10 min. At this point, the microtubule-binding fragment became soluble, and lane 4 of Figure 2 demonstrates that we have achieved homogeneity using HPLC separation with a reverse-phase C18 column. It should be noted that our overall purification scheme has proven to be equally efficient for over 30 different site-specific mutant forms of the microtubule-binding region.

Interaction of MAP-2 MTBR with Tubulin and Assembled MTs. Sloboda *et al.* (1976) demonstrated that MAP-2 readily promotes tubulin self-assembly with half-maximal stimulation occurring in the 2−5 μM range, and later work with thrombin cleavage to produce the microtubule-binding fragment of MAP-2 (Joly *et al.*, 1989) showed that this fragment and intact high-molecular-weight MAP-2 bound to microtubules with virtually identical affinity ($K_d = 2-5$ μM). The data shown in Figure 3 indicate that the bacterially expressed microtubule-binding fragment MAP-2 *MTBR* stimulates tubulin polymerization. Indeed, while self-assembly of pure tubulin (10 μM) proceeded very slowly under these conditions, we observed that the polymerization rate was increased by more than a factor of 50 at 3.2 μM MAP-2 *MTBR*, and the extent of polymerization was also greatly enhanced. As shown in Figure 4A, the initial plateau values for polymerization depended upon the concentraton of MAP-2 *MTBR* in a linear fashion. In companion experiments (Figure 4B), we varied the tubulin concentration in the presence of the bacterial expression product to obtain estimates of the critical tubulin concentration required for microtubule polymerization. We found that microtubules polymerized in the presence of MAP-2 *MTBR* exhibited a critical concentration of 2 μM tubulin. We should note that we used 3.4 μM levels of the recombinant protein in these experiments, and such

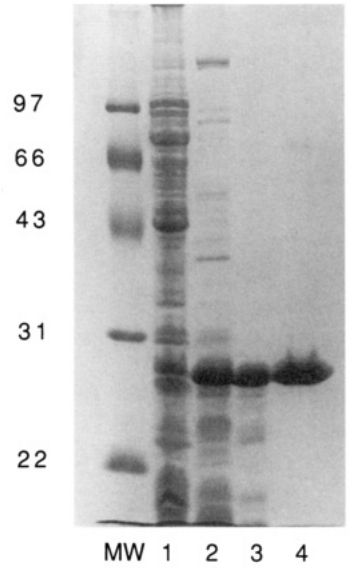

FIGURE 2: SDS gel electrophoretic analysis of bacterially expressed MAP-2 *MTBR* at various stages of isolation: cell-free extract (lane 1); insoluble fraction collected by centrifugation through a sucrose cushion and resuspended by homogenization (lane 2); protein after heat treatment and clarification by ultracentrifugation (lane 3); and MAP-2 *MTBR* after reverse-phase HPLC fractionation (lane 4). (Note: molecular weight standards are shown at left.)

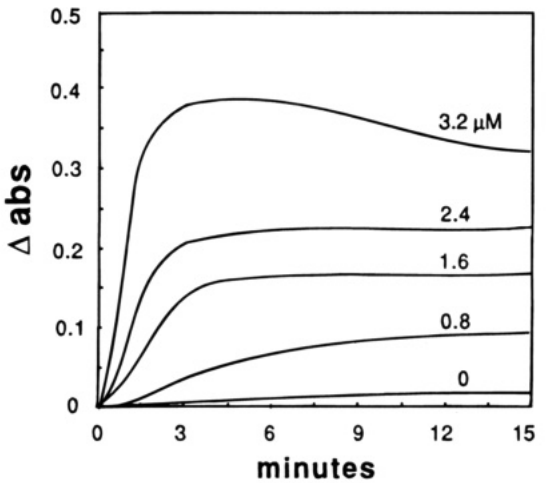

FIGURE 3: Microtubule assembly induced by the recombinant MAP-2 microtubule-binding region. Assemblies were carried out with 10 μM tubulin at 37 °C and followed by turbidity at 350 nm. The recombinant MAP-2 fragment was at concentrations ranging from 0 to 3.2 μM.

concentrations would correspond to that observed in 2.5 mg/mL recycled microtubule protein (*i.e.*, tubulin and MAPs).

As an additional test of the reversible binding of MAP-2 *MTBR*, we also conducted competitive binding experiments with the MAP-2 second repeated sequence peptide analogue (**VTSKCGSLKNIRHRPGGGRVK**) using taxol-stabilized microtubules and subsequent ultracentrifugation to separate microtubule-bound and free MAP (Joly & Purich, 1990). As shown in lanes 1 and 2 of Figure 5, MAP-2 *MTBR* was present in both supernatant and pellet fractions, as would be expected when MAPs are in excess concentration relative to tubulin (*i.e.*, 10 and 2.5 μM, respectively). However, at 0.4 mM (lanes 3 and 4) and 2.0 mM (lanes 5 and 6) of the repeated sequence analogue, virtually all of the microtubule-binding domain can be displaced from assembled microtubules.

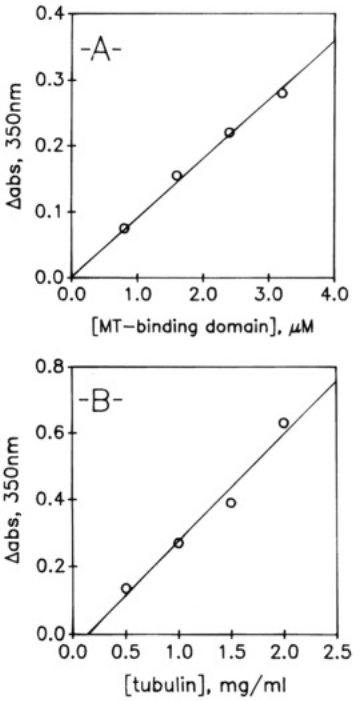

FIGURE 4: Extent of tubulin polymerization in the presence of bacterially expressed MAP-2 fragments. (A) Plot of the overall amplitude of microtubule assembly data (from Figure 3) *versus* the concentration of the microtubule-binding domain. (B) Critical behavior of microtubules assembled with MAP-2 *MTBR*. Samples of assembled microtubules were subjected to various extents of dilution in the presence of the recombinant protein, and turbidity measurements were made to estimate the amount of microtubule polymer remaining assembled. Tubulin (20 μM) was assembled by addition of MAP-2 fragment maintained at 3.4 μM, and dilutions were made as indicated while holding the binding domain concentration at 3.4 μM.

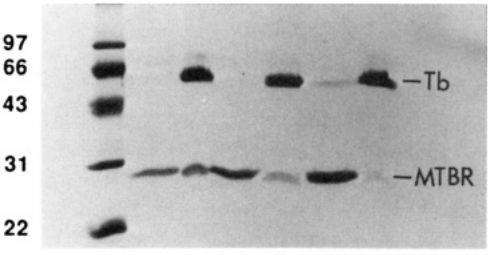

FIGURE 5: Displacement of MAP-2 *MTBR* from taxol-stabilized microtubules by a 21-amino acid peptide (m_2') corresponding to the second nonidentical repeat sequence of MAP-2. The amount of microtubule-binding domain remaining associated with microtubules was visualized by SDS gel electrophoresis after separation of microtubule pellet and supernatant fractions obtained by ultracentrifugation through a sucrose cushion (20% w/v). Molecular weight markers are indicated at the left, and lanes 1−2, 3−4, and 5−6 correspond to pairwise supernatant and pellet fractions obtained in the presence of 0, 0.4, and 2.0 mM peptide m_2', respectively.

Mutations in the First and Third Nonidentical Repeats of the MT-Binding Region. Earlier findings from our laboratory (Joly *et al.*, 1989; Joly & Purich, 1990; Yamauchi *et al.*, 1993) suggest that the second repeated sequence may be the primary site on MAP-2 for MT binding. Nonetheless, those earlier studies with 18- and 21-amino acid peptide analogues were inconclusive because the analogues of the second repeat displayed 60−100× higher dissociation constants for MT binding when compared to MAP-2 or its thrombin-cleaved MT-binding region. Accordingly, we have conducted site-

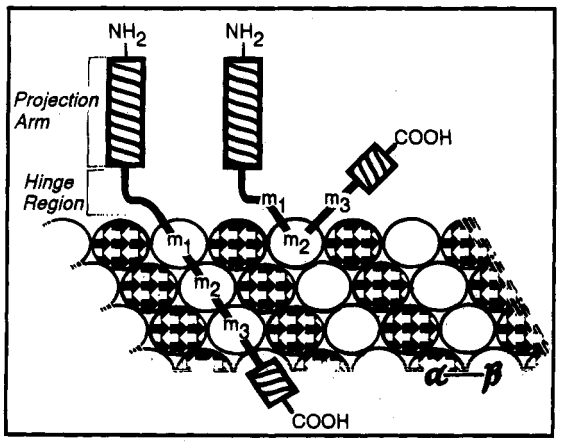

FIGURE 6: Two models for repeated sequence interactions with microtubules. Tubulin α and β subunits are shown as darkened and open circles, respectively. The binding scheme at the left indicates the multisubunit interaction, and the scheme at the right involves a single interaction with the second repeat.

directed mutagenesis experiments in which the first and third repeats were modified to resemble the second repeat. If one considers the interactions diagrammatically presented in Figure 6, then MAP-2 binding to MTs can be depicted as a single associative interaction between the second repeat (or m_2) and a single MT site or as a multisite interaction involving the additive effects of all three repeated peptide sequences (*i.e.*, m_1, m_2, and m_3). If the single-site case applies, then no significant improvement in the strength of MAP binding should occur as a result of mutations in the first or third repeats, even if they are modified to more closely resemble the second repeated sequence. If the multisite case is operant, then a substantial increase in MAP affinity should attend the conversion of either the first alone, the third alone, or their combined replacement. Intermediate levels of affinity would be expected if the first and second or the second and the third repeats interacted with the microtubule.

Accordingly, we constructed and expressed the following mutant MT-binding regions: MAP-2 *MTBR*[$m1_2$-m_2-m_3], MAP-2 MTBR[m_1-m_2-m_{32}], and MAP-2 *MTBR*[m_{12}-m_2-m_{32}], and each has the entire sequence from position 1629 (thrombin site) to position 1828 (C-terminus). The subscripts "12" and "32" indicate that the m_1 and m_3 sequences have been modified (with specific substitutions shown in boldface letters) to resemble m_2 (see Figure 7). Joly *et al* (1989) showed that while peptides m_1 and m_3 failed to stimulate *in vitro* tubulin polymerization, the m_2 peptide was effective. We did not make complete replacements of m_1 and m_3 sequences by m_2, and we therefore conducted a series of experiments using synthetic peptide analogues corresponding to peptides m_{12} and m_{32} to confirm that they behaved in a manner analogous to m_2. As shown in Figure 7, synthetic peptides designated m_{12} and m_{32} were as effective as peptide m_2 in displacing the microtubule-binding region from taxol-stabilized MTs. This observation confirms the feasibility of using these altered peptide sequences to mimic the second repeat sequence. We then proceeded to characterize the MT-binding properties of the aforementioned mutant MT-binding domains (Figure 8). In these experiments, we evaluated the strength of microtubule binding by determining the amount of MTBR displacement from taxol-stabilized microtubules as a function of the concentration of the 21-amino acid m_2' peptide VTSKCGSLKNIRHRPGGGRVK, which contains

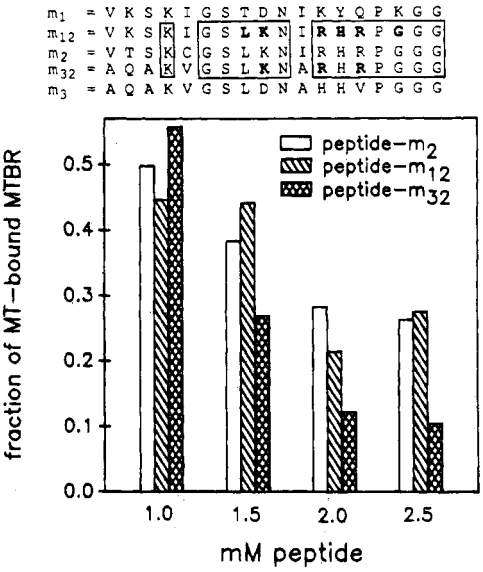

FIGURE 7: Displacement of MAP-2 *MTBR* from taxol-stabilized MTs by synthetic octadecapeptides corresponding to m_2-like sequences substituted for the first and third repeats in the expression plasmids described in the text (peptide m_{12} and peptide m_{32}, respectively). Above are the sequences for the modified repeat peptides (m_{12} and M_{32}) aligned with the three nonidentical repeats in the MAP-2 *MTBR*. Residues in boldface represent changes made in the first and third sequences to render tham more like the second MAP-2 repeat, particularly with respect to positively charged side-chain groupings. Tubule-bound and unbound MAP-2 *MTBR* fractions were separated by ultracentrifugation. Pellet fractions were then subjected to SDS–polyacrylamide gel electrophoresis followed by Coomassie staining. The amount of MT-binding domain in each pellet fraction was measured by gel densitometry and was normalized relative to the density of the MAP-2 *MTBR* band obtained in the absence of any competitor peptide.

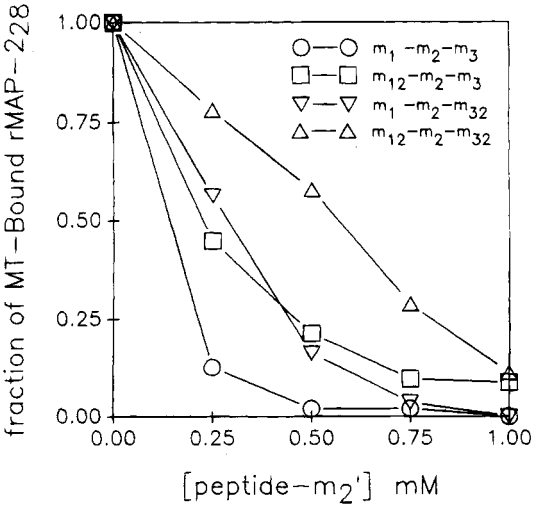

FIGURE 8: Displacement of MT-binding domain mutants and wild-type MAP-2 *MTBR* (m_1-m_2-m_3) by peptide m_2'. Mutants m_{12}-m_2-m_3, m_1-m_2-m_{32}, and m_{12}-m_2-m_{32} correspond to changes in the first and third nonidentical repeats to resemble the second repeat (see text). Tubule-bound and unbound MAP-2 *MTBR* were separated by ultracentrifugation. Pellet fractions were subjected to SDS–polyacrylamide gel electrophoresis followed by Coomassie staining. The amount of MT-binding domain in the pellet fraction was measured by densitometry of the gels and is normalized relative to the density of the MAP-2 *MTBR* band obtained in the absence of peptide m_2'.

additional positive charge at the C-terminal end and offers the advantage that one can work at lower peptide concentrations in displacement assays (Joly & Purich, 1990). We

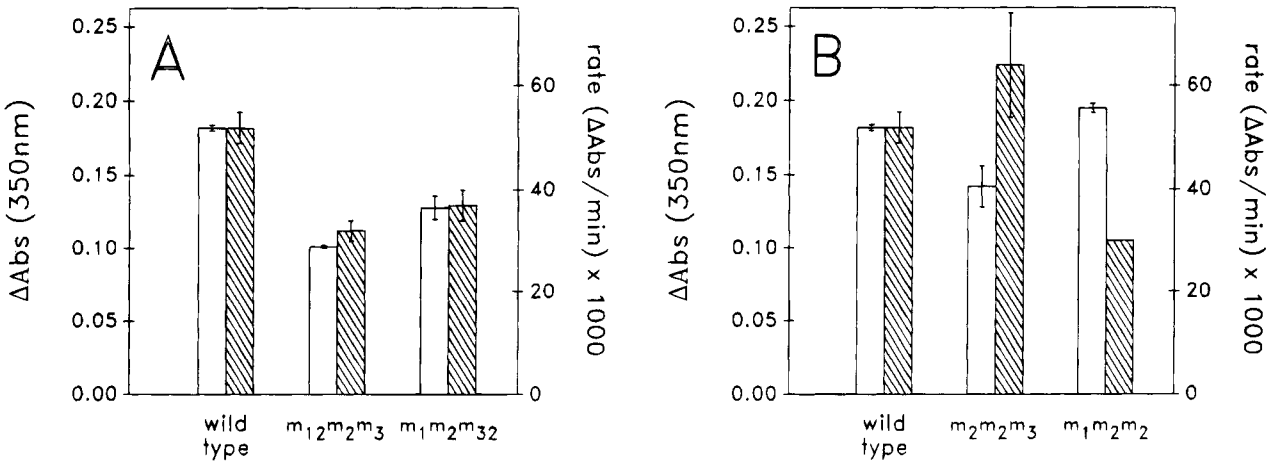

FIGURE 9: Extent and rate of tubulin polymerization induced by mutant and wild-type forms of MAP-2 *MTBR*. (A) Wild-type and mutants with either the first or the third repeat changed to resemble the second repeat. (B) Wild-type and mutants with the first or third repeat replaced with an identical copy of the second repeat. Duplicate assembly runs were made for each at 37 °C with 10 μM tubulin and 1 μM MTBR. The error bars represent the range. Extent (indicated by the open bars) and rate (indicated by hatched bars) of tubulin polymerization are averages of these duplicates.

found that the wild-type binding fragment displayed weaker binding than MAP-2 *MTBR*[m_{12}-m_2-m_3] or MAP-2 *MTBR*[m_1-m_2-m_{32}], and all three bound less strongly than the combined mutant MAP-2 *MTBR*[m_{12}-m_2-m_{32}]. In terms of the concentration of peptide m_2' required to displace half of th binding fragment from taxol-stabilized microtubules, the wild-type and combined mutant MAP-2 *MTBR*[m_{12}-m_2-m_{32}] gave values of 0.13 and 0.6 mM, respectively. This degree of enhanced binding falls considerably short of that expected if all three repeated sequences bind simultaneously on a microtubule, and these data suggest that the second repeated sequence is the primary site of interaction. In support of this conclusion is our separate finding that complete substitution of the m_2 region with m_3, using cassette mutagenesis, greatly reduced the extent of binding. Indeed, in assembly studies with comparable levels of wild-type and this mutant binding domain, we observed that the extents of assembly after 25 min in the presence of 10 μM tubulin were 0.205 and 0.028, respectively (R. L. Coffey and D. L. Purich, manuscript in preparation). The latter value is only slightly above that which we typically observe for tubulin self-assembly in the absence of microtubule-associated protein.

Sloboda, Dentler, and Rosenbaum (1976) first demonstrated that microtubule-associated proteins can greatly stimulate tubule polymerization, presumably by stabilizing nucleation as well as by reducing the critical concentration for microtubule self-assembly. Both the rate of polymerization and also the extent of polymerization, respectively, can be used as indices for evaluating MAP interactions with microtubules. Thus, as an independent test of the contribution of each peptide repeat to MAP binding, we studied the ability of mutant forms of the MT-binding region to stimulate microtubule assembly. Shown in Figure 9A are the rates and extents of MAP-stimulated tubulin polymerization for wild type, MAP-2 MTBR[m_{12}-m_2-m_3], and MAP-2 *MTBR*[m_1-m_2-m_{32}]. These data demonstrate that conversion of the first repeat or third repeat to resemble the second repeat has little effect on the ability of MAPs to stimulate assembly. Furthermore, we tested two additional mutant forms: the first, designated as MAP-2 *MTBR*[m_2-m_2-m_3], has the first repeat replaced by an exact m_2 octadecapeptide repeat; and the second mutant form, designated MAP-2 *MTBR*[m_1-m_2-m_3], has the third repeat replaced by an identical m_2 sequence. The findings presented in Figure 9B indicate that the position of additional second peptide repeats has some statistically distinguishable effect on the rate of tubulin polymerization, and future work will be needed to address whether this is an effect on nucleation or elongation kinetics. As stated earlier, if multiple binding of m_2-like sequences occurred, one should anticipate that the additivity of free energies of binding should be reflected in a substantial multiplicative change in the apparent affinity of a MAP for microtubule-binding sites. Here, MAP mutants containing extra copies of the second repeat sequence would be expected to be far more effective in promotiong tubule assembly, and the data in Figure 9B clearly indicate that the contrary is true.

DISCUSSION

In the present report, we describe the purification and preliminary characterization of bacterially expressed MAP-2 MT-binding region starting from the thrombin cleavage site (numbered 1629 in the mouse sequence) to the C-terminus. Our focus on the microtuble-binding region of this brain-specific MAP relates to a general interest in defining the subsite interaction that stabilize fibrous MAP binding to microtubules. It is noteworthy that this region retains all of the binding properties of adult MAP-2ab (Joly *et al.*, 1989), and bacterial expression and purification of wild-type and mutant binding regions now open the way for a more detailed biochemical and structural inquiry. Likewise, these developments should facilitate future experiments with living cells to understand structure/function relationships affecting the molecular design of this neuronal cytomatrix protein.

Earlier sequencing of mouse and rat MAP-2 cDNA, respectively, revealed that both proteins have highly conserved regions in their extreme amino and carboxyl regions (Lewis *et al.*, 1988; Kindler *et al.*, 1990), and our studies with bovine MAP-2, a protein from an evolutionarily more distant organism, indicate the remarkable preservation of sequence throughout the microtubule-binding domain. Indeed, from position 1538 to the C-terminus at position 1828, there are only two substitutions (*i.e.*, an D/E at 1564 and a G/S at 1785) and a three amino acid HTP insert in the bovine sequence immediately following the thrombin cleavage site

at position 1629. This HTP insert is also present in the human MAP-2 sequence (Albala *et al.*, 1993). Such extensive preservation of sequence has been observed in only a few cases; most notable is the HSP70 multigene family of heat shock proteins (Hendrick & Hartl, 1993), including the endoplasmic reticulum protein BiP and the major constitutively expressed hsc70 proteins. While these are arguably the most conserved sequences known, differing only by 1 or 2 substitutions in their 650 residues, our observations with the microtubule-binding region also point to strong evolutionary pressure to maintain structure in this region of MAP-2. One might expect that such conservation reflects the stability against mutation displayed by other proteins with which MAP-2 interacts, and the tubulins are widely recognized for their evolutionary stability. Studies with MAP-2 and related work with tau and MAP-4 indicate that the tubule-binding domain is primarily confined to the repeated sequence region may allow tau to bundle microtubules. Of course, proteins other than tubulin may interact with MAP-2, and in this context, the functional significance of the sequence preservation will require further investigation. Flynn *et al.* (1987), for example, observed that the MT-binding region binds to neurofilaments, and scanning mutagenesis studies may provide a means for defining the locus of this complexation reaction.

The present investigation has also allowed us to study the microtubule-binding properties of site-specific mutant forms of the MAP-2 MT-binding region. We have found that mutations in the first and third repeated peptide sequences can lead to somewhat higher affinity microtubule binding when such sequences are made to resemble the m_2 subsite. It should be noted, however, that Goode and Feinstein (1994) recently demonstrated the importance of so-called inter-repeat regions that intervene between each of the nonidentical repeats in tau protein, and future work must address the importance of corresponding structural elements in MAP-2. Furthermore, as we gain additional insight regarding MAP−microtubule interactions by making other single or multiple point mutations in the second repeat, we may be able to enhance binding even further. Earlier studies demonstrated that the inclusion of three additional amino acids (RVK) to the synthetic octadecapeptide m_2 greatly improved the ability of the analogue to stimulate microtubule assembly and to displace high-molecular-weight MAP-2 from microtubules (Joly & Purich, 1990). When one considers that the m_1 and m_3 sequences correspondingly have QVQ and NVK following their sequences, it is reasonable to anticipate that the introduction of additional positive charge by replacement with RVK should also enhance binding to microtubules. Indeed, many earlier studies have demonstrated that ionic charge is likely to be an important driving force in the complexation of MAP-2 with microtubules (Bryan, 1976; Yamauchi & Purich, 1987). Such high-affinity mutant forms could be especially valuable in manipulating microtubule interactions in neurons where MAP-2 is thought to cross-link microtubules through the projection-arm region.

All available evidence points to a dominant role of the second repeated sequence in MAP-2 binding to microtubules, which in Figure 6 is illustrated in terms of β-subunit binding based on the findings of Cross *et al.* (1990). First, peptide analogues of this subsite promote tubulin polymerization, whereas analogues to the other repeats do not (Joly *et al.*, 1989). Second, only m_2 peptide analogues displace MAP-2 (Joly & Purich, 1990). Third, Dingus *et al.* (1991) demonstrated that antibody binding to the third repeat does not interfere with MAP-2 binding to microtubules, indicating that the repeated sequence makes no significant contribution to the binding energy. Fourth, that replacement of m_2 by the corresponding m_3 sequence results in almost complete loss of ability to stimulate tubule asembly also accords with this conclusion. While improvement in the microtubule binding affinity can be accomplished by conversion of the first and third sequences to resemble m_2, the modest gain can be accounted for by assuming that only one sequence is actually engaged in microtubule binding and that we have effectively increased the molarity of m_2-like sequences by a factor of 3. If the m_2 and the additional m_2-like sequences had interacted at three MT sites simultaneously, the binding energy ($\Delta G_{overall}$) would be the summation of the individual binding energies (ΔG_i). The Gibbs equation [$\Delta G_{overall} = -nRT \ln K$] predicts that any additivity in binding energy will have an exponential effect on the observed value of K. Hence, if each additional repeat sequence interaction augmented the binding energy by only 50%, the observed effect on K would be a factor of 30. This was not observed in our experiments where we obtained an improved binding corresponding to a factor of 3−4 at most, corresponding to a very small incremental change in $\Delta G_{overall}$. In sum, we are drawn to the single m_2 interaction model in Figure 6, and additional work will be required to discern whether the flanking inter-repeat sequences contribute further to binding energy.

Although this second repeat region is quite important in MAP-2 interactions with MTs, we cannot exclude the participation of other subsites within the microtubule-binding region. Experiments with protein kinase C phosphorylation of MAP-2 indicate that residues immediately preceding and following the second repeat must contribute to the overall binding interaction (Ainsztein & Purich, 1994). Indeed, phosphorylations at S1703 (located in the first inter-repeat). S1711 (positioned in the second repeat), and S1728 (located in the second inter-repeat) are all involved in modulating the binding of MAP-2 to microtubules. These findings are in harmony with the conclusion of Butner and Kirschner (1991) that the binding energy for tau protein interactions with MTs is delocalized and derives from a series of weak interactions on the part of small groupings of amino acids. It should be noted that tau proteins and MAP-2 share many common features within the *MTBR* region, with the notable exception that the first nonidentical repeat in tau can itself promote tubulin polymerization while this is not the case for the MAP-2 first repeat. Butner and Kirschner (1991) suggest that the higher relative affinity of the first tau repeat indicates that is may serve to anchor tau protein to the microtubule while the more weakly interacting remaining repeats release about once each millisecond (if addition is diffusion-limited). Although their model suggests that tau protein may assume a variety of bound positions on the microtubule lattice, there is no direct experimental method currently available for establishing or refuting such a proposal.

The availability of the MAP-2 microtubule-binding domain should now permit detailed characterization of the subsite interactions within the repeated peptide sequences as they relate to microtubule binding. This should also permit us to address such related issues as (a) the location of phos-

phorylation sites that may be important in controlling MAP-2 interactions with microtubules and/or other cytomatrix components, (b) the nature of phospholipid interactions, particularly phosphatidylinositol to MAP-2 (Yamauchi & Purich, 1987, 1993), and (c) the nature of MAP-2 binding interactions with neurofilaments, which are thought to occur through the binding domain region (Flynn *et al.*, 1987). We should also be able to utilize site-directed mutagenesis to rearrange the order and spacing of the three repeated sequences in the microtubule-binding region of MAP-2.

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