Members of the heat-stable family of microtubule-associated proteins (MAPs), MAP 2, tau, and MAP 4, contain three or four tandem imperfect repeated sequences close to their carboxyl termini. These sequences lie within the microtubule-binding domains of the MAPs; they have been proposed to be responsible for microtubule binding and the ability of these MAPs to lower the critical concentration for microtubule assembly. Their spacing may reflect that of the regularly arrayed tubulin subunits on the microtubule surface. We here characterize the 32- and 34-kDa chymotryptic microtubule-binding fragments of MAP 2 identified in earlier work. We identify the primary chymotryptic cleavage site in high molecular weight MAP 2 as between Phe^{1253} and Lys^{1256}, within 13 amino acids of the known MAP 2 splice junction. We have raised a monoclonal antibody to the 32- and 34-kDa fragments and find that it reacts with all members of the heat-stable MAPs class. To determine where it reacts, we sequenced immunoreactive subfragments of the 32- and 34-kDa fragments, selected several cDNA clones with the antibody, and tested for antibody reactivity against a series of synthetic MAP 2 and tau peptides. We identify the epitope sequence as HHVPGGG (His-His-Val-Pro-Gly-Gly-Gly). The antibody also recognized several other MAP 2 and tau repeats. Despite reacting with this highly conserved element, we find that the antibody does not block microtubule binding, but binds to the MAPs and co-sediments with microtubules. These results suggest that there are other regions besides the repeated elements which are essential for microtubule binding.

Microtubule-associated proteins (MAPs) are a diverse group of proteins involved in regulating microtubule assembly and function. A subset of MAPs is heat-stable, and this class includes the neuronal proteins MAP 2 and tau, and the more widespread MAP 4 (for reviews, see Olmsted, 1986; Vallee, 1990; Lee, 1990). These MAPs have multiple isoforms, all with a microtubule-binding domain near the carboxyl terminus. The function of the remainder of the protein, which for MAP 2 and MAP 4 is most of the molecule, is largely unknown, but may involve interactions with other proteins. For example, MAP 2 has a binding site near its amino terminus for the RII subunit of protein kinase A (Obar et al., 1989; Rubino et al., 1989).

The three types of heat-stable MAP have related sequences within their microtubule-binding domains. All of these proteins contain similar 18-amino acid sequences which are repeated with a periodicity of ~31 amino acids (Lee et al., 1988; Lewis et al., 1989; Aizawa et al., 1990). These elements have a relatively high percentage of basic amino acids (pI values in the range of 10 to 11). Tau has isoforms with either three or four repeats (Himmler, 1989; Goedert et al., 1989). MAP 2 has three repeats (Lewis et al., 1988), and MAP 4 has both three and four repeat isoforms (Chapin and Bulinski, 1991).

Bacterially expressed MAP 2 fragments containing all three or just the first two repeats were found to bind to microtubules through cycles of assembly and disassembly (Lewis et al., 1988). Tau isoforms with four repeats were better at promoting microtubule assembly than those with three repeats (Goedert and Jakes, 1990). Tau constructs with two repeats (the first and fourth, or the first and third) did not bind as well to microtubules (Himmler, 1989; Lee et al., 1989), and constructs with only one repeat (the fourth) bound weakly (Lee et al., 1989). The microtubule-binding and assembly-promoting properties of the repeats were also tested using synthetic peptides (Ennulat et al., 1989; Joly et al., 1989; Joly and Purich, 1990). These studies found that the individual repeats have distinct effects on microtubules. For tau, only peptides with the first and third repeat sequences were found to promote microtubule assembly, but at a concentration ~1000-fold higher than that required with tau. For MAP 2, only the peptide with the second repeat sequence was found to bind to microtubules and promote assembly. These studies indicate that while the repeat has the properties of a microtubule-binding unit, a single repeat is insufficient for normal microtubule binding.

As an alternative means of investigating the mechanism of microtubule binding by the heat-stable MAPs, we have produced a monoclonal antibody to the microtubule-binding region of MAP 2. We report here that the antibody cross-reacts with all of the heat-stable MAPs and that the epitope lies within the third repeat sequence of MAP 2. To our knowledge, this is the first example of an antibody specific for a class of MAPs. However, the antibody does not inhibit microtubule binding, a finding which has important implications for the role of the repeats in the interaction of the heat-stable MAPs with microtubules.

**MATERIALS AND METHODS**

*Purification of Proteins and Protein Fragments—Calf brain microtubules were prepared by the reversible assembly method, and tubulin*
was purified from these microtubules as described (Vallee, 1986). Heat-stable brain MAPs were prepared from thrice-cycled microtubules by addition of salt to 0.75 M and dithiothreitol to 10 mM, incubation at 100 °C for 5 min, and centrifugation to remove coagulated protein. Tau, MAP 2C, and high molecular weight MAP 2 were separated chromatographically on Sepharose CL-4B (Sigma) in 0.75 M NaCl in PEM buffer (100 mM Pipes, 1 mM EGTA, pH 6.7, 1 mM MgSO₄). All proteins were dialyzed into PEM buffer prior to use. Microtubule-binding fragments of MAP 2 were prepared as previously described (Vallee, 1986). Rat liver microtubules were prepared using taxol (Collins and Vallee, 1989), and rat liver MAP 2 was prepared from DEAE-purified tubulin (5 mg/ml in PEM buffer) by the addition of 20 μM taxol and incubation at 37 °C for 15 min and were added to a final concentration of 1 mg/ml (~20 μM tubulin), and the samples were incubated for an additional 30 min at 37 °C. Samples were centrifuged through 10% sucrose cushions in PEM buffer containing 10 μM taxol and 1 mM GTP at 18,000 rpm for 30 min at 37 °C. Pellets were resuspended to the original sample volume, and supernatants and pellets were analyzed on SDS-polyacrylamide gels followed by staining with Coomassie Blue. Densitometry (Quick-Scan Jr., Helena Industries) was performed in order to determine the relative percentages of protein in the supernatants and pellets.

Brain microtubules were prepared by three cycles of polymerization and depolymerization in PEM buffer with 1 mM GTP and stabilized by the addition of 20 μM taxol, then diluted to a final concentration of 3 mg/ml. Antibody was included at 1–5 μM. The samples were incubated for 2 h at 37 °C, then centrifuged through sucrose, and analyzed as above.

A bacterially expressed four-repeat isoform of tau (htau40) was purified as described (Goedert and Jakes, 1990). Two micromoles were incubated at 23 °C in the presence and absence of antibody, then rebound to taxol-stabilized microtubules, and analyzed as described above.

An antibody of thrice-cycled microtubules was monitored by the increase in turbidity at 320 nm, using a Gilford Model 250 spectrophotometer. Samples of microtubule protein, with or without antibody, were incubated on ice for 1 h, then transferred to a water-jacketed cuvette (NSG Precision Cells, Farmingdale, NY) connected to a temperature-controlled circulating water bath. Assembly was initiated by warming the sample to 37 °C. The microtubule samples were also sedimented and subjected to polyacrylamide gel electrophoresis; the amounts of protein in each sample were quantitated by densitometry.

RESULTS

An Antibody to the Microtubule-binding Domain of Heat-stable MAPs Reacts within the Imperfect Repeat Sequences—Antibody MAP 2-4 was raised to be used as a probe for studying the mechanism of microtubule binding by MAP 2. As can be seen in Fig. 1, the antibody reacted with the 32- and 34-kDa chymotryptic microtubule-binding fragments of MAP 2 (Vallee, 1980) (Fig. 1, lanes 2 and lanes 4), which were used as immunogen, and with intact high molecular weight MAP 2 (Fig. 1, lanes 1). The antibody also reacted with...
microtubule-binding fragments of approximately 25 kDa. No reaction was observed with non-microtubule-binding fragments of MAP 2 (Fig. 1B, lane 3).

Antibody reaction was also observed in the undigested microtubule sample with a series of endogenous high molecular weight MAP 2 fragments routinely seen in microtubule and MAP 2 preparations. Additional reactivity was observed with a polypeptide of \(~70\) kDa, the electrophoretic position of MAP 2C (Fig. 1B, lane 1). The amount of this species present is variable and seems to correlate with the age of the calf brain used for these experiments; more is present in microtubules from the brains of younger calves. This is consistent with the pattern of developmental expression of MAP 2C described for the rat protein (Riederer and Matus, 1985).

Purified calf brain tau was also found to react with antibody (Fig. 2A); all the tau bands were stained with the antibody. This appears to explain the immunoreactive band seen migrating just ahead of tubulin in Fig. 1B (lane 1), where the position and appearance of the multiple tau bands were affected by the large amount of tubulin. To determine if MAP 4 is also cross-reactive with the antibody, rat liver microtubules were immunoblotted. As can be seen in Fig. 2B, cross-reaction with MAP 4 was observed. No reaction was seen with any of the other MAPs in our preparations, including MAP 1A and 1B (Bloom et al., 1984), cytoplasmic dynein (Paschal et al., 1987), kinesin (Vale et al., 1985), dynamin (Shpetner and Vale, 1989), and the MAP1 light chains (Hammaback et al., 1991)).

To define the epitope sequence recognized by antibody MAP 2-4, protein chemical and molecular cloning methods were used. Amino-terminal sequencing of the 32- and 34-kDa microtubule-binding fragments of MAP 2 was performed to localize these species within the MAP 2 molecule (Fig. 3). The two fragments were determined to have identical amino-terminal sequences. The sequence was compared to that of mouse MAP 2 (Lewis et al., 1988) and was found to align with it beginning at amino acid 1526.

The 32- and 34-kDa microtubule-binding fragments were further digested with chymotrypsin or V-8 protease, and subfragments immunoreactive with the antibody were identified. These were sequenced and found to derive from the region of the third repeat element in MAP 2 (Fig. 3 and Table I). The 32 residues of bovine sequence obtained from these subfragments are identical with the corresponding mouse sequence (amino acids 1732-1764) and comprise the entire third repeat sequence.

The antibody cross-reactivity with tau suggested that the epitope for the antibody lay directly within the repeats. To test this hypothesis, we examined antibody binding to synthetic peptides corresponding to the repeats in MAP 2 and tau (Figs. 4 and 5). The interaction was tested by two different methods. In the first, the peptides were bound to a solid matrix (Immobilon membranes) and then challenged with the antibody. The strongest reaction was observed with the m3 peptide (corresponding to the third MAP 2 repeat). A weaker reaction was variably observed with the m2 peptide, which has the sequence of the second MAP 2 repeat (data not shown). No reaction was seen with the m1 peptide. Peptides corresponding to all but the second of the four tau repeats were available; these three peptides were all reactive, but the t1 peptide (corresponding to tau repeat 1) reacted less strongly than did the other tau peptides.

The second method used to examine antibody-peptide interaction was to look at inhibition by the peptides of antibody binding to MAP 2 and tau. The antibody and peptides were incubated together in solution, then these mixtures were tested for reaction with MAP 2 and tau. The results, shown in Fig. 5, are similar to those of Fig. 4. Only the m3, t3, and t4 peptides inhibit the antibody binding to MAP 2 and tau, respectively. (The m2 and t1 peptides have little effect in this experiment, and this probably reflects a much lower affinity of the antibody for these sequences.) A very high peptide concentration, in the millimolar range, was required in order to significantly inhibit antibody reaction with intact protein. In a separate experiment, it was determined that the antibody has a higher affinity for the third MAP 2 repeat sequence than for any of the tau sequences (data not shown).

The limits of the epitope within the third repeat sequence were defined following the analysis of several cDNA clones selected using the antibody from rat brain \(\lambda\)gt11 expression libraries. The derived protein sequences of these clones were very different from those of the MAP repeats except for a very short sequence in each, as shown in Table I. Based on these data and the results of the synthetic peptide experiments (Figs. 4 and 5 and summarized in Table I), we conclude that the epitope for antibody MAP 2-4 is the sequence HHVPGGG, the last 7 amino acid residues in m3. All the other sequences that bind the antibody are similar, with only 1 or 2 residues changed or deleted. The m3 peptide (corresponding to the third MAP 2 repeat), of course, has this sequence. The tau peptides that bind the antibody well (t3 and t4, corresponding to the third and fourth repeats of tau) contain only one change from the above epitope sequence: HHKPGGG for t3 and THVPGGG for t4. We note that this region also corresponds to the most highly conserved region within the imperfect repeats.

**Antibody MAP 2-4 Binds to Heat-stable MAPs on Microtubules**—In view of the prevailing hypothesis that the repeats are the microtubule-binding sites and reflect the interaction of the MAPs with the regularly spaced tubulin subunits on the microtubule surface, we examined the effect of the antibody on the interaction of MAPs and microtubules. Fig. 6, A and B, shows the results of including the antibody during the rebinding of MAP 2 to taxol-stabilized microtubules. A polyacrylamide gel of these samples is shown in Fig. 6A. The MAP 2, tubulin, and antibody light chain bands are clearly visible; the antibody heavy chain is largely obscured by the tubulin band, and only a minor heavy chain component can be seen, migrating slightly behind the tubulin. No change in

**Fig. 2. Monoclonal antibody MAP 2-4 cross-reacts with tau and MAP 4.** In panel A, calf brain tau was run on a 10% SDS gel, transferred to nitrocellulose, and challenged with antibody; lane 1 is the Coomassie Blue-stained gel, and lane 2 is the immunoblot. In panel B, rat liver microtubule protein was run on a 10% SDS gel, transferred, and immunoblotted; lanes 1 and 2 are the Coomassie Blue-stained gel, lanes 3 and 4 are the immunoblot. Lanes 1 and 3 are the heat-stable MAP 4 preparation from the microtubules, and lanes 2 and 4 are the heat-denatured fraction. Molecular weights are shown \(\times 10^3\).
Antibody to Microtubule-binding Domain

The gel was scanned, and the percentage of antibody light chain in the supernatants was determined. The amount of antibody in the pellets increased only slightly, indicating a finite number of binding sites. The antibody did not saturate the reconstituted microtubules; the amount of MAP 2 in the microtubule preparations; the amount of MAP 2 present here is between 0.8 and 6 μM. Instead, the antibody partitioned between the supernatant and the microtubule pellet (as best judged from the behavior of the antibody light chain). With increased amounts of antibody added, the amount of antibody in the pellet increased only slightly, indicating a finite number of binding sites. The antibody did not sediment with MAP-free microtubules (lanes 6 and 12). The gel was scanned, and the percentage of antibody light chain in the supernatants versus pellets was determined. These data are expressed graphically in Fig. 6B; saturating levels of antibody in the pellets are reached at about 0.8 μM, which is close to the concentration of MAP 2, 1.1 μM. This would correspond to a stoichiometry of one antibody molecule for every MAP 2 molecule on the microtubules. (The ratio of MAP 2 to tubulin in these reconstituted pellets is similar to that of cyclic brain microtubules.)

The antibody also binds to microtubules from cycled preparations with MAPs already present (3 mg/ml total microtubule protein). Fig. 7A shows the increasing amount of antibody sedimenting with the microtubules versus the amount of antibody added to the original sample. Again, as in Fig. 6, the amount of antibody found in the microtubules at saturation is 2.5 μM, approximately equal to the molar concentration of MAP 2 in the microtubule preparations; the amount of MAP 2 present here is between 2 and 3 μM. In Fig. 7B, lanes 1 and 2 are the supernatant and pellet of a control with antibody only, and lanes 3 and 4 are antibody with MAP-free microtubules. Both demonstrate that in the absence of MAPs, very little antibody sediments. Next are supernatants and microtubule pellets from two of the samples from Fig. 7A: 1 μM and 5 μM antibody. When less than stoichiometric amounts of antibody are added (1 μM), nearly all the antibody bound (lanes 5 and 6); at 5 μM antibody, the microtubules are saturated with antibody and as much is seen in the supernatant as in the pellet.

As shown in Fig. 8, a four-repeat form of bacterially expressed tau bound to microtubules (lanes 3 and 4) with no detectable interference by the antibody (lanes 5 and 6 and 7 and 8), even though the antibody reacts with at least two sites (the third and fourth repeats) in this form of tau. However, the antibody did not saturate the reconstituted microtubules at the same ratio as with MAP 2, but at a ratio of approximately 1 antibody molecule for every 2 tau molecules, which probably reflects the lower affinity of the antibody for the binding sites in tau.

The effect of the antibody was also examined by light sedimenting with the microtubules that of cycled brain microtubules.)

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**Fig. 3. Linear model of bovine MAP 2 with location of peptide sequences.** Some known features of MAP 2 are shown: the repeats in the microtubule-binding domain (near the carboxyl terminus) and the RII binding region. Arrowheads indicate the splice sites for MAP 2C. Above the model, the sequence from the two antibody-binding peptides is given; the bracket indicates the sequence of the third MAP 2 repeat, m3. The amino-terminal sequence of the microtubule-binding MAP 2 fragments is given below the model. In each case, the underlined portion is the duplicated sequence; in parentheses, the number of the 1st residue in each sequence, using the numbering scheme of mouse MAP 2 (Lewis et al., 1988); starred residues differ from this sequence. The scale indicates the amino acid residue numbers.

**Table 1: Antibody MAP 2-4 binding sequences and related sequences**

| Sequence                        | 2-4 binds? |
|---------------------------------|------------|
| Bovine MAP 2 CT-20:             | +++        |
| Bovine MAP 2 V8-14:             | +++        |
| Murine MAP 2 repeat 3 (m3):     | +++        |
| Murine MAP 2 repeat 2 (m2):     | +/−        |
| Murine MAP 2 repeat 1 (m1):     | −          |
| Murine tau repeat 1 (t1):       | +          |
| Murine tau repeat 3 (t3):       | +++        |
| Murine tau repeat 4 (t4):       | +++        |
| λgt11-LD:                       | +++        |
| λgt11-RS:                       | +++        |

the amount of MAP 2 in the supernatants (lanes 1 through 5) or pellets (lanes 7 through 11) was seen as the amount of antibody increased from 0 to 6 μM. Instead, the antibody partitioned between the supernatant and the microtubule pellet (as best judged from the behavior of the antibody light chain). With increased amounts of antibody added, the amount of antibody in the pellet increased only slightly, indicating a finite number of binding sites. The antibody did not sediment with MAP-free microtubules (lanes 6 and 12). The gel was scanned, and the percentage of antibody light chain in the supernatants versus pellets was determined. These data are expressed graphically in Fig. 6B; saturating levels of antibody in the pellets are reached at about 0.8 μM, which is close to the concentration of MAP 2, 1.1 μM. This would correspond to a stoichiometry of one antibody molecule for every MAP 2 molecule on the microtubules. (The ratio of MAP 2 to tubulin in these reconstituted pellets is similar to that of cyclic brain microtubules.)

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The effect of the antibody was also examined by light sedimenting with the microtubules that of cycled brain microtubules.)

**Fig. 3. Linear model of bovine MAP 2 with location of peptide sequences.** Some known features of MAP 2 are shown: the repeats in the microtubule-binding domain (near the carboxyl terminus) and the RII binding region. Arrowheads indicate the splice sites for MAP 2C. Above the model, the sequence from the two antibody-binding peptides is given; the bracket indicates the sequence of the third MAP 2 repeat, m3. The amino-terminal sequence of the microtubule-binding MAP 2 fragments is given below the model. In each case, the underlined portion is the duplicated sequence; in parentheses, the number of the 1st residue in each sequence, using the numbering scheme of mouse MAP 2 (Lewis et al., 1988); starred residues differ from this sequence. The scale indicates the amino acid residue numbers.

**Table 1: Antibody MAP 2-4 binding sequences and related sequences**

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|---------------------------------|------------|
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| Bovine MAP 2 V8-14:             | +++        |
| Murine MAP 2 repeat 3 (m3):     | +++        |
| Murine MAP 2 repeat 2 (m2):     | +/−        |
| Murine MAP 2 repeat 1 (m1):     | −          |
| Murine tau repeat 1 (t1):       | +          |
| Murine tau repeat 3 (t3):       | +++        |
| Murine tau repeat 4 (t4):       | +++        |
| λgt11-LD:                       | +++        |
| λgt11-RS:                       | +++        |
scattering (turbidimetry), as measured by the optical density at 320 nm (Fig. 9). When microtubules were assembled in the presence of antibody, the turbidity of the assembled microtubules in solution approximately doubled (Fig. 9C). However, under these conditions, there was no change in the amount of sedimentable tubulin or MAP 2 (Fig. 9, A and B). Thus, the turbidity increase must reflect either an increase in the light-scattering of the microtubules due to the added mass of bound peptide or a change in the microtubule sedimentation coefficient.

**Fig. 4.** Antibody MAP 2-4 was used to detect MAP 2, tau, and peptides slot-blotted onto Immobilon P. The left-most column, m, has the peptides m1, m2, and m3, which have the sequence of the first, second, and third MAP 2 repeats, respectively. The center column, t, has the peptides t1, t3, and t4, which have the sequence of the first, third, and fourth tau repeats, respectively. The right-most column, c, has the controls, purified MAP 2, tau, and tR, a randomized sequence with the amino acid composition of t. The amount of protein or peptide added to each slot is given on the far left (in picomoles, pm, and nanomoles, nm). In column C, the unlabeled slots are blanks. The arrows indicate MAP 2 and m3 peptide at approximately the same concentration; the arrowheads indicate the same for tau and all three tau peptides. Antibody concentration is ~1 µM.

**Fig. 5.** Antibody MAP 2-4 binding to MAP 2 and to tau is inhibited by a subset of repeat peptides. Purified antibody was preincubated with varying concentrations of peptides, then challenged with electroblotted strips of MAP 2 (panel A) or tau (panel B). Peptides used in panel A are labeled as in Fig. 4: m1, m2, and m3; control without peptide is at left, labeled c. From left to right within each bracket, concentrations of peptide are: 5 µM, 50 µM, 500 µM, and 5 mM. Antibody is approximately 1 nM. Peptides used in panel B are also labeled as in Fig. 4: t1, t3, t4, and tR. c is control without peptide. From left to right within each bracket, concentrations of peptide are: 50 µM, 500 µM, and 5 mM. Antibody concentration is ~10 nM.

**Fig. 6.** Antibody MAP 2-4 binds to MAP 2 on microtubules. In panel A, heat-stable brain MAPs (predominantly MAP 2) were preincubated with purified antibody MAP 2-4, then rebound to microtubules. Five different concentrations of antibody were used, at a constant microtubule concentration of 1 mg/ml (10 µM tubulin dimer) and 1.1 µM MAP 2. Lanes 1 through 6 are the supernatants, and lanes 7 through 12 are the pellets containing the microtubules. Lanes 1 and 7, 0 antibody; lanes 2 and 8, 1.5 µM antibody; lanes 3 and 9, 3 µM antibody; lanes 4 and 10, 4.5 µM antibody; lanes 5 and 11, 6 µM antibody; lanes 6 and 12, 1.5 µM antibody without MAPs. 50, molecular weight × 10⁻⁶, the approximate position of tubulin and the antibody heavy chain; Ab lc, antibody light chain. The amount of antibody in the supernatants and pellets was quantitated and expressed in panel B as a graph: the concentration of antibody in the resuspended microtubule pellet versus the total concentration of antibody in the starting sample.

**Fig. 7.** Antibody MAP 2-4 binds to MAP 2 on cycled microtubules. Panel A is a graph of antibody concentration in the microtubule pellet versus the concentration of antibody in the starting sample; six different concentrations of antibody were used. SDS-gel analysis of some of the samples is shown in panel B. Lanes 1 and 2 show the supernatant and pellet of an antibody only control; lanes 3 and 4 show antibody plus MAP-free microtubules control; lanes 5 and 6 show 1 µM antibody; lanes 7 and 8 show 5 µM. The concentration of microtubule protein was 3 mg/ml. 50, molecular weight × 10⁻³, the approximate position of tubulin and antibody heavy chain; AB lc, antibody light chain.
antibody plus MAP-free microtubules; concentration of tau is 2 antibody heavy chain; and absence of antibody; shown is the SDS gel of these samples.

change during microtubule assembly (data not shown), probably for the same reasons.

Antibody MAP 2-4 binds to tau on microtubules. Bacterially expressed tau was bound to microtubules in the presence of antibody, or possibly to cross-linking or bundling of the microtubules (although no bundles were seen when antibody-treated microtubules were examined in the electron microscope). The antibody also increased the rate of turbidity change during microtubule assembly (data not shown), probably for the same reasons.

DISCUSSION

We have succeeded in producing a monoclonal antibody to the tandem repeats in the microtubule-binding domain of MAP 2. The antibody has proven to be the first example of a MAP class-specific probe; that is, it reacts with all of the known mammalian heat-stable MAPs, including the high and low molecular weight forms of MAP 2, tau, and MAP 4. No reaction is observed with MAPs which are unrelated in primary sequence to the heat-stable MAPs. These include MAP 1B heavy chain (Noble et al., 1989), MAP 1B light chain 1 (Hammarback et al., 1991), the kinesin heavy chain (Yang et al., 1989), and dynamin (Obar et al., 1990). Nor does the antibody react with MAP 1A (Bloom et al., 1984) or with MAP 1C/cytoplasmic dynein (Paschal et al., 1987), suggesting that these proteins also differ from the heat-stable MAPs in their microtubule-binding domains.

Amino-terminal sequencing of the 32- and 34-kDa chymotryptic microtubule-binding fragments of MAP 2 has determined that the chymotryptic cleavage site is on the amino-terminal side of Lys150 (using the numeration scheme for mouse high molecular weight MAP 2, Lewis et al., 1988). This site is 13 amino acids from the known splice junction of the lower molecular weight form of MAP 2, MAP 2C. (The only species for which an exact splice junction is known is the rat, but since the rat, mouse, and our limited bovine sequences are all nearly identical, we assume that the splice junctions in all three species are analogous. In the rat, the MAP 2C splice junction is on the 5' side of nucleotide 4603 and corresponds to the codon for amino acid residue 1514 (Kindler et al., 1990a, 1990b).) The proximity of the chymotryptic cleavage site to the splice junction suggests that this region represents a domain boundary.

Antibody-binding subfragments of these 32- and 34-kDa fragments were found to derive from the third repeat sequence in the microtubule-binding domain of MAP 2 (see Fig. 3).

Comparison of the sequence of the subfragments, synthetic repeat peptides, and antibody-binding fusion proteins led to the identification of the sequence HHVPGGG as the epitope sequence, corresponding to the last 7 amino acid residues of the third MAP 2 repeat motif. This is in the most highly conserved region within the repeats, suggesting that it is functionally, as well as evolutionarily, important.

The antibody only exhibited strong binding with the third...
repeat peptide, m3 (Figs. 4 and 5); weak and variable reactivity was seen with the m2 peptide and none with the m1 peptide. With the tau peptides, good binding was seen with both the t3 and t4 peptides; a weaker reaction was seen with the t1 peptide. These other antibody-binding sequences do not have the exact epitope sequence HHVPGGG, but are very similar with one or two amino acid changes. Indeed, the antibody appears to have a slightly lower affinity for tau than for MAP 2, in spite of the multiple binding sites in tau. A peptide corresponding to the second tau repeat, present only in adult forms of the protein, was not available; however, we predict that it should bind to the antibody, as it contains the sequence KHQPGGG, which has only one change from the epitope sequence. Therefore a four-repeat form of tau should have three antibody binding sites. Fig. 10 summarizes all these results and shows the location of the epitope and the other binding sites for this antibody. We also predict that there are two sites in MAP 4 to which the antibody binds, and that they are in the first and second repeats of MAP 4. The sequences are KHQPGGG and KHQPGGG, respectively, and each has two amino acid substitutions from the epitope sequence.

Although it is reasonable to expect that an antibody to this region of MAP 2 (or tau) would interfere with microtubule binding, the antibody does not block the binding of either MAP to microtubules. It is clearly interacting with the microtubules, since it co-sediments with microtubules in a saturable manner and increases the turbidity of the microtubules in solution.

Our data are consistent with those of Joly et al. (1989) and Joly and Purich (1990) who found that a synthetic peptide corresponding to the m2 repeat stimulated microtubule assembly. Peptides corresponding to m1 and m3 had no detectable effect. Since our antibody reacts most strongly with m3 and only weakly with m2, it is possible that the persistent microtubule binding by MAP 2 that we observe in the presence of antibody reflects the activity of the m2 sequence. However, this is unlikely since m2 alone binds ~1,000 times less strongly to microtubules than does MAP 2.

In the case of tau, synthetic peptides corresponding to the t1 and t3 repeats were found to promote microtubule assembly in vitro (Ennulat et al., 1989). Our antibody showed good binding to t3 and t4 (Figs. 4 and 5), and, as noted above, we predict binding to t2 as well. Since tau bound to microtubules in the presence of antibody, it is possible that the first tau repeat alone may be sufficient for microtubule binding. Again, this would not be inconsistent with the peptide data, except that t1 alone had far lower microtubule assembly promoting activity than did tau itself.

Even considering that the antibody does not interact with all of the MAP 2 and tau repeats, the IgG molecule is relatively large on the size scale of tubulin and the MAPs. It is logical, therefore, to expect some steric inhibition of the interaction of the MAP with the microtubule. Yet, in repeated experiments, the antibody caused no detectable decrease in the amount of MAP bound to the microtubules.

One possible explanation for this is the existence of microtubule-binding elements in addition to the three imperfect repeats. In MAP 2, a domain boundary may lie 150 amino acids upstream of the first repeat sequence, as suggested by the location of the MAP 2C splice junction and the primary chymotryptic cleavage site (Fig. 3). It is tempting to speculate that the microtubule-binding domain of MAP 2 extends to this boundary. This region of MAP 2 is also very basic, and comparable regions upstream of the tau and MAP 4 repeats are also basic. Thus, it is possible that the microtubule-binding domain of the heat-stable MAPs may be more complex than current models predict. Nonetheless, the conservation and organization of the repeat sequences implies some essential role in microtubule assembly.

Acknowledgments—We wish to thank Dr. Dona Chikaraishi for the gift of rat brain cDNA library, Dr. Christine Collins for liver microtubules, Dr. Ronald Liem for the gift of the tau repeat peptides, and Dr. Daniel Purich for the gift of the MAP 2 repeat peptides. We thank the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for providing taxol. We also thank Dr. John Hildebrandt for critical reading of the manuscript and Louise Ohrn for excellent technical assistance.

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