Analysis of the Microtubule-binding Domain of MAP-2

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ABSTRACT We examined the microtubule-binding domain of the microtubule-associated protein (MAP), MAP-2, using rabbit antibodies that specifically bind to the microtubule-binding region ("stub") and the projection portion ("arm") of MAP-2. We found that (*a*) microtubules decorated with arm antibody look similar to those labeled with whole unfractionated MAP antibody, though microtubules are not labeled with stub antibody; (*b*) incubation of depolymerized microtubule protein with stub antibody prior to assembly partially inhibits the rate of microtubule elongation, presumably because MAPs that are complexed with antibody cannot bind to microtubules and stabilize elongating polymers; (*c*) the rate of appearance and amounts of 36- and 40-kD microtubule-binding peptides produced by digestion with chymotrypsin are distinct for MAPs associated with microtubules vs. MAPs free in solution. The enhanced stability of the 40-kD peptide when associated with microtubules suggests that this domain of the protein is closely associated with, or partially buried in, the microtubule surface; (*d*) MAP-2 is a slender, elongate molecule as determined by unidirectional platinum shadowing (90 ± 30 nm), which is in approximate agreement with previous observations. Stub antibody labels MAP-2 in the terminal one-quarter of the extended protein, indicating an intrinsic asymmetry in the molecule.

Microtubules isolated from brain tissue by cycles of temperature-dependent assembly and disassembly contain tubulin and several microtubule-associated proteins (MAPs)[1], including MAP-1 (300 kD), MAP-2 (280 kD), and tau (55–70 kD), which co-purify with tubulin and promote microtubule assembly and stabilization in vitro (Berkowitz et al., 1977; Borisy et al., 1975; Herzog and Weber, 1978; Murphy and Borisy, 1975; Sloboda et al., 1976; Weingarten et al., 1975). MAP-2 comprises more than half of the nontubulin protein in microtubules prepared by in vitro assembly.

By platinum shadowing, thin sectioning, and negative staining methods, MAP-2 has been shown to project outward from the microtubule wall (Voter and Erickson, 1982; Zingsheim et al., 1979; Amos, 1977; Dentler et al., 1975; Kim et al., 1979; Murphy and Borisy, 1975). Cleavage of microtubules containing MAPs with alpha chymotrypsin produces a 35–40-kD microtubule-binding domain which binds to microtubules and stimulates assembly, as well as a 240-kD projection portion which lacks microtubule-binding activity (Vallee and Borisy, 1977). However, the exact size of the microtubule-binding region of MAP-2 and its position relative to tubulin subunits in the microtubule wall have not yet been determined.

MAP-2 has also been shown to be bound to microtubule surfaces in a regular manner, there being a 32-nm periodic spacing between adjacent MAPs on the lateral surfaces of microtubules. Amos (1977) proposed that the pattern of MAP attachment is helical and may be related to the helical arrangement of tubulin subunits in the microtubule wall. However, Voter and Erickson (1982), using platinum shadowing, did not observe a regular distribution of MAPs on microtubules, and both Sheterline (1978) and Gottlieb and Murphy (1983) observed a 100-nm repeat interval using MAP antibody labeling procedures. Therefore, there is not a consistent picture regarding the details of the pattern of MAP-2 attachment on microtubules.

In an effort to obtain more detailed information about the distribution of MAPs on microtubules and especially about the location and size of the microtubule-binding domain of MAP-2, we used antibodies specific to different portions of MAP-2. By affinity chromatography of polyclonal rabbit antibodies to MAP-2, we obtained one fraction that specifically labels the 36–40-kD microtubule-binding portion of MAP-2 ("stub") and another antibody fraction that recognizes the projection portion of MAP-2 ("arm"). In this paper we present

[1] *Abbreviations used in this paper:* MAP, microtubule-associated protein; MTP, microtubule protein.

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our findings using these polyclonal affinity-purified antibodies to stub and arm domains of MAP-2.

MATERIALS AND METHODS

Preparation of Cycled Microtubule Protein and MAP-2

Microtubule protein was purified from hog brain by two cycles of in vitro assembly and disassembly in 0.1 M Na-PIPES buffer at pH 6.94 by a modification of the method of Borisy et al. (1974) as described by Murphy (1982). For most experiments, microtubule pellets which had been stored at −80°C were thawed, resuspended in assembly buffer (0.1 M Na-PIPES, pH 6.94, containing 0.1 mM MgCl and 1.0 mM GTP) and passed through a third cycle of temperature-dependent assembly–disassembly to prepare a microtubule protein (MTP). Fractions of purified MAP-2 were prepared by heat treatment (Fellous et al., 1977) followed by gel filtration chromatography on 4% agarose.

Preparation of MAP Arm and MAP Stub Affinity Columns

Hog brain MTP at 3 mg/ml in assembly buffer containing 10% glycerol was polymerized with taxol (30 μM), pelleted through 25% sucrose cushions containing taxol, and resuspended to 3 mg/ml in assembly buffer with taxol. To generate MAP arm and stub fragments, alpha-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) was added (0.1 μg/1.0 mg MTP, 5 min, 37°C), the reaction was arrested with 5 mM phenylmethylsulfonyl fluoride, and the microtubules were removed by pelleting through 25% sucrose cushions.

MAP ARM AFFINITY COLUMN: MAP "arms" released by treatment with chymotrypsin were precipitated with 50% ammonium sulfate, resuspended in water, and centrifuged briefly to remove insoluble aggregates. The soluble material was fractionated by gel filtration chromatography (BioGel A15m) to isolate the large proteolytic fragments (140 and 180 kD) corresponding to "arm" (column buffer: 50 mM imidazole, 50 mM KCl, 10 mM beta-mercaptoethanol, 0.5 mM MgCl, 0.1 mM EDTA, pH 7.0). To prepare an affinity column for arm-specific antibodies, we selected fractions which excluded intact MAP-2 and smaller fragments the same size as the "stub." The fractions containing "arm" were concentrated by ammonium sulfate precipitation and then linked to cyanogen bromide–activated Sepharose by standard methods (Porath, 1974).

MAP STUB AFFINITY COLUMN: The microtubule pellet obtained after chymotryptic digestion was resuspended in assembly buffer with taxol (10 μM), incubated at 37°C for 20 min, and made 0.35 M in NaCl according to the method of Vallee (1982) to release MAP stubs from the microtubules. The microtubules were pelleted by centrifugation, and the stub-containing supernate was dialyzed against ammonium bicarbonate (pH 7.0), lyophilized, and resuspended in water. This material was loaded on a 12% preparative SDS polyacrylamide gel, electrophoresed, and lightly stained (10 min in 1% Coomassie blue, then destained in water) to visualize the bands. The zone containing the bands corresponding to 35–40-kD stub fragments was cut out from the gel and the stub peptides were eluted by electrophoresis (Stephens, 1975), concentrated by dialysis, and attached to cyanogen bromide–activated Sepharose.

Preparation of MAP Antibodies

Rabbits were immunized with heat-soluble fractions of MAP-2 (Fellous et al, 1977; Kim et al., 1979) that were purified by gel filtration chromatography and combined with Bentonite by the method of Gallily and Garvey (1968). Antiserum was examined for specific antibody by a solid-phase binding assay using the procedure of Tsu and Herzenberg (1980) as modified by Kiehart et al. (1984). We used polystyrene microtiter plates and ^{125}I-labeled protein A to quantitate the amount of antibody binding. The specificity of antibody fractions was determined by examining the binding of proteins that were fractionated by SDS gel electrophoresis and transferred by electrophoretic blotting onto nitrocellulose sheets by the method of Towbin et al. (1979). Strips labeled with ^{125}I-protein A were dried, and autoradiograms were prepared to identify immunoreactive components.

Antibodies specific to MAP-2 were purified on an affinity column prepared from cyanogen bromide–activated Sepharose containing MAP-2 as described by Porath (1974), and eluted by a modification of the procedure described by Bennett and Stenbuck (1979). In earlier work, this antibody has been shown to recognize MAP-2 and its proteolytic fragments, but not tubulin or tau (Gottlieb and Murphy, 1983).

To purify antibody against the arm component of MAP-2, antiserum was passed over an affinity column containing stub antigen. The flow-through from this column (containing IgG which did not bind stub antigen) was then passed over an affinity column containing arm antigen, eluted with 1.0 M acetic acid, partially neutralized to pH 4.0 with 1 M Tris, dialyzed against PBS, and stored at −80°C until use. This material is referred to as "arm" antibody.

To purify antibody against the stub component of MAP-2, the flow-through fraction from an arm affinity column (IgG which did not bind arm antigen) was passed over a stub affinity column and eluted and treated as described above. This preparation is referred to as "stub" antibody.

Protein A–Gold Colloid Preparation

Reduction of gold chloride by sodium borohydride to form the colloid was performed according to the method of Rash et al. (1982). Protein A was adsorbed onto the activated colloid by a modification of the procedure described by Horisberger and Rosset (1977). Protein-coated colloid was fractionated on glycerol gradients to obtain particles with an average diameter of 10–20 nm as determined by electron microscopy. For antibody labeling experiments, protein A–gold colloid was used at a concentration giving an optical density of 0.33 at 520 nm. We found by evaluation of grids containing decorated microtubules that this concentration gave optimal labeling of microtubules relative to the background.

Antibody Labeling of Microtubules

Microtubule protein was polymerized at 37°C in assembly buffer with 10 μM taxol for 15 min and then diluted to 0.25 mg/ml in pre-warmed buffer containing 1% glutaraldehyde. After 2 min, 3 μl were deposited on a carbon-coated grid, treated three times with 20 mM sodium borohydride for 4 min each time to inactivate residual aldehyde groups (Osborn and Weber, 1982), rinsed three times with phosphate-buffered saline (PBS) for 3 min each time, incubated with MAP-2 antibody (0.1 mg/ml in PBS) for 30 min, rinsed twice with PBS for 3 min each time, incubated with protein A–gold colloid for 15 min, rinsed twice again with PBS for 3 min each time, and negatively stained with 1% aqueous uranyl acetate.

Platinum Shadowing of MAP-2 and MAP-2 Stub Antibody Complexes

MAP-2 prepared as described above was diluted to 20 μg/ml in distilled water and 15% glycerol, sprayed onto freshly cleaved mica, and vacuum dried at room temperature using a Polaron evaporator for up to 5 h, or until the vacuum was better than 5×10^{-7} torr (Hall, 1956; Fowler and Erickson, 1979; and Voter and Erickson, 1982). The dried specimens were then shadowed unidirectionally at a 6° angle. A carbon coating deposited directly over the specimen stabilized the metal replicas, which were then floated off onto a distilled water surface and picked from beneath with 400 mesh copper grids. After the grids were dried they were examined in a Zeiss EM 10A electron microscope.

To visualize stub antibody bound to MAP-2, we incubated MAP-2 at 130 μg/ml with stub antibody at 63 μg/ml for 60 min at 37°C (molar ratio of ~1:1). To overcome surface tension effects which tended to disrupt the antigen–antibody complexes, we diluted this solution 100-fold in 20% glycerol and water, and sprayed the mica sheets heavily so that the droplets coalesced to form a thin film of solution over the entire mica sheet. We found this to yield a better distribution of material with greater preservation of antigen–antibody complexes than other methods. Samples were vacuum dried and shadowed as described above. Random fields of the electron microscopy grids were examined and every antigen–antibody complex observed was photographed at a magnification of 50,000×.

Turbidimetric Assays of Microtubule Assembly

Cycled microtubule protein was cold-depolymerized, sonicated, and centrifuged at 200,000 *g* for 2 h at 5°C to remove rings. This high-speed supernate was diluted to 1 mg/ml and incubated for 15 min at 0°C with PBS, preimmune IgG, MAP-2 antibody, arm antibody, or stub antibody at 0.05 or 0.5 mg/ml. The sample was incubated for 45 s in a water-jacketed cuvette to allow complete equilibration to assembly temperature (30°C). Polymerized, sheared microtubules (seeds) were added to a final concentration of 0.17 mg/ml and turbidity changes were recorded at 350 nm. Initial rates of polymerization obtained from the slopes of the recordings were corrected (+10%) to account for slight differences in the amount of seeds added to the cuvettes.

PAGE and Determination of Protein Concentration

SDS slab gels containing 12% acrylamide were prepared by the method of Laemmli (1970) with the modification of Anker (1970). Gels were stained with Coomassie Blue and destained as described by Fairbanks et al. (1971). Protein was applied to gels in amounts known to fall within the linear range of staining intensity with Coomassie Blue (Borisy et al, 1975).

Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Characterization of Affinity-Purified Antibodies to MAP Arm and Stub

We purified antibodies to the microtubule-binding portion (stub) and projection portion (arm) of MAP-2 as described in Materials and Methods. The MAP antibody is specific for MAP-2 and does not recognize either MAP-1 or tau (not shown). The relative specificities of arm and stub antibodies were examined by immunoblotting (Fig. 1). Arm antibody (*A* and *B*) recognizes a large class of high molecular weight fragments and after double-column treatment (*B*) the reactivity to stub (*B*, lane *3*) is almost entirely removed. The intensity of staining of arm fragments (lane *2*) and intact MAP (lane *1*) remains about the same.

It can be seen that stub antibody (*C* and *D*) binds strongly to the 35–40-kD stub (lane *3*) and to intact MAP (lane *1*) but also binds to high molecular weight cleavage products present in the arm fraction (lane *2*). The reactivity to fragments in the arm fraction probably represents the presence of high molecular weight fragments that contain part of the stub region. Such fragments could be produced if chymotrypsin cleaved dissociated MAPs at secondary sites. Although the stub and arm antibodies showed a certain degree of nonspecificity by immunoblotting, in practice, both antibodies proved to be highly specific for morphologic analyses and studies of microtubule assembly (see Discussion).

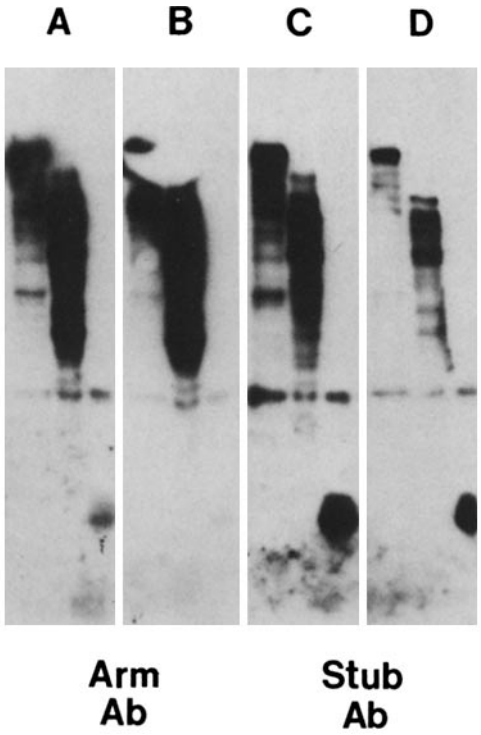

FIGURE 1 Immunoblot demonstrating reactivities of rabbit antibodies against intact MAP-2, arm, and stub antigens. The affinity purification of arm- and stub-specific antibodies by single-column and double-column purification methods is described in Materials and Methods. In each panel, lane 1 contains intact MAP-2, lane 2 contains arm antigen, and lane 3 contains stub antigen. (*A* and *B*) Arm antibody after single-column (*A*) and double-column purification (*B*). (C and *D*) Stub antibody after single-column (C) and double-column purification (*D*).

Since the stub antigen used in preparing the affinity column was SDS denatured, we first determined if the antibody from this column was able to recognize native (nondenatured) stub. Native stub was obtained by the methods of Vallee and Borisy (1977), and Vallee (1982): microtubules treated with alpha-chymotrypsin were pelleted and stubs released from the taxol-stabilized microtubules by resuspension in buffer containing high salt were isolated from the supernate following a second centrifugation to remove microtubules. A side-by-side comparison of native stub and intact MAP-2 by a solid-phase binding assay confirmed that the stub antibody was capable of recognizing native stub as well as intact MAP-2 (data not shown).

Antibody Decoration of Intact Microtubules

Glutaraldehyde-fixed microtubules were labeled with stub antibody, arm antibody, or whole (unfractionated) MAP-2 antibody, followed by decoration with protein A–gold, and negatively stained for examination by electron microscopy. Microtubules were densely and uniformly labeled with tubulin antibody (Fig. 2*D*), but were not labeled with preimmune IgG (Fig. 2*E*), indicating that microtubules are specifically labeled by this procedure. Microtubules containing MAPs were also heavily labeled after incubation with unfractionated MAP antibody (Fig. 2*A*) or with arm antibody (Fig. 2*B*). The presence of IgG–protein A–gold complexes away from the microtubule wall is consistent with the finding that MAP-2 projects away from microtubule surfaces (compare with the pattern of labeling with tubulin antibody in Fig. 2*D*). Incubation with stub antibody, however, gave minimal or no labeling, (Fig. 2*C*), suggesting that the stub region may not be accessible to antibody while bound to microtubules.

Antibody Labeling of Microtubules Digested with Chymotrypsin

Microtubules digested with chymotrypsin (0.1 μg/mg MTP) sufficient to remove arms (electron microscopic analysis) and cleave >90% of the MAP-2 band (gel analysis) can still be labeled with MAP antibody (Fig. 3*B*). In an earlier report we concluded that the labeling obtained after digestion with 0.1 μg chymotrypsin may be due to the binding of MAP antibody by the remaining MAP stubs (Gottlieb and Murphy, 1983). However, the finding described in the previous section, that stub antibody does not bind to microtubules, prompted us to examine the disposition of the stub on microtubule surfaces in more detail. To achieve a more complete digestion we therefore used a higher enzyme concentration (0.2 μg/mg MTP). Coomassie Blue–stained gels of microtubules treated in this way indicated loss of intact MAP-2, the presence of 35–40-kD stub, and varying quantities of high molecular weight fragments (data not shown). These microtubules could no longer be labeled with any of the MAP antibody fractions

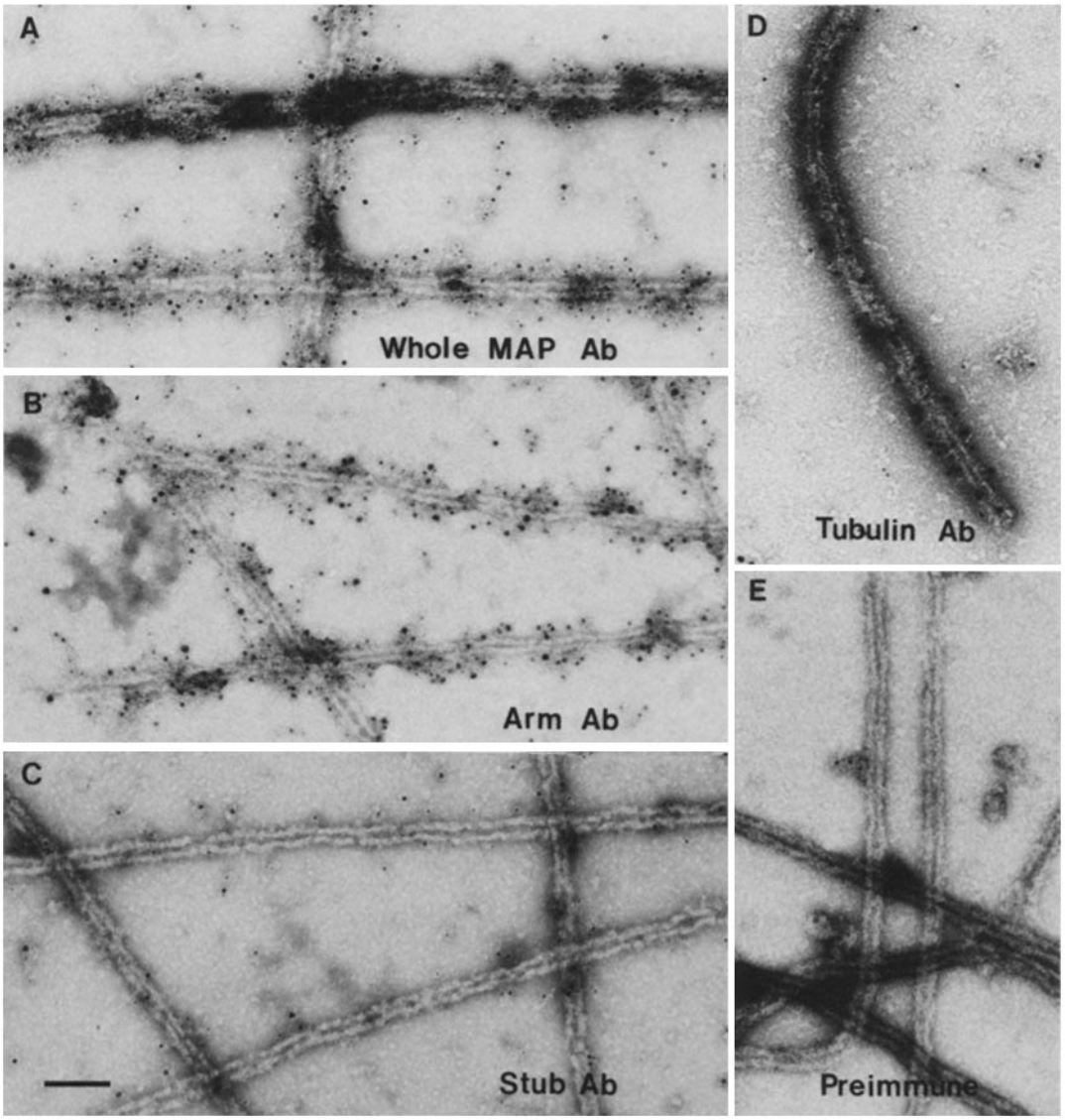

FIGURE 2 Electron micrographs of microtubules labeled with rabbit polyclonal antibodies and protein A–gold, and negatively stained with uranyl acetate. (*A*) Microtubules labeled with affinity-purified MAP-2 antibody (but not fractionated into arm and stub fractions) are heavily labeled. (*B*) Microtubules incubated with arm antibody are also extensively decorated. (*C*) Labeling microtubules with stub antibody causes little or no decoration. (*D*) Tubulin antibody gives dense, uniform labeling. (*E*) Preimmune IgG from the rabbit from which MAP-2 antibodies were obtained does not label microtubules. ×104,000.

(Fig. 3*C*). This result is consistent with the lack of decoration seen with stub antibody (Fig. 2*C*) and suggests that the 35–40-kD stub portion of MAP-2 is not accessible to antibody while bound to microtubules.

Stub Antibody Inhibits Microtubule Assembly

To prove that the stub antibody in fact bound to the microtubule-binding domain of MAP-2, we examined the effects of MAP antibodies on microtubule assembly. Table I shows the inhibition we observed when antibodies were preincubated with soluble microtubule protein at 0°C prior to elongation on microtubule seeds. The details of the procedure are described in Materials and Methods. The results of two series of experiments, designated series *A* and *B*, are shown. The addition of preimmune IgG at 0.05 mg/ml (*A*) had little effect on polymerization, although it was somewhat inhibitory at 0.5 mg/ml (*B*). This was true generally for IgG fractions prepared from rabbit serum. MAP antibody resulted in a reduced amount of polymerization in series *A* but gave rise to increased turbidity in series *B*; however, electron microscopic inspection of this sample revealed cross-linked microtubules and immune complexes, not seen in the other samples. Arm antibody had an effect not much greater than that of preimmune serum. In both series, stub antibody was effective in partially inhibiting polymerization. This presumably is due to the fact that stub antibody specifically binds to the microtubule-binding portion of MAP-2 and thereby prevents its association with microtubules. It is curious that the rate of elongation was not inhibited to an even greater extent, but it is possible that microtubule elongation is effectively stimulated by even small amounts of MAPs.

We found that microtubule self-assembly was not inhibited by stub antibody when antibody was added to depolymerized microtubule protein containing ring oligomers before polym-

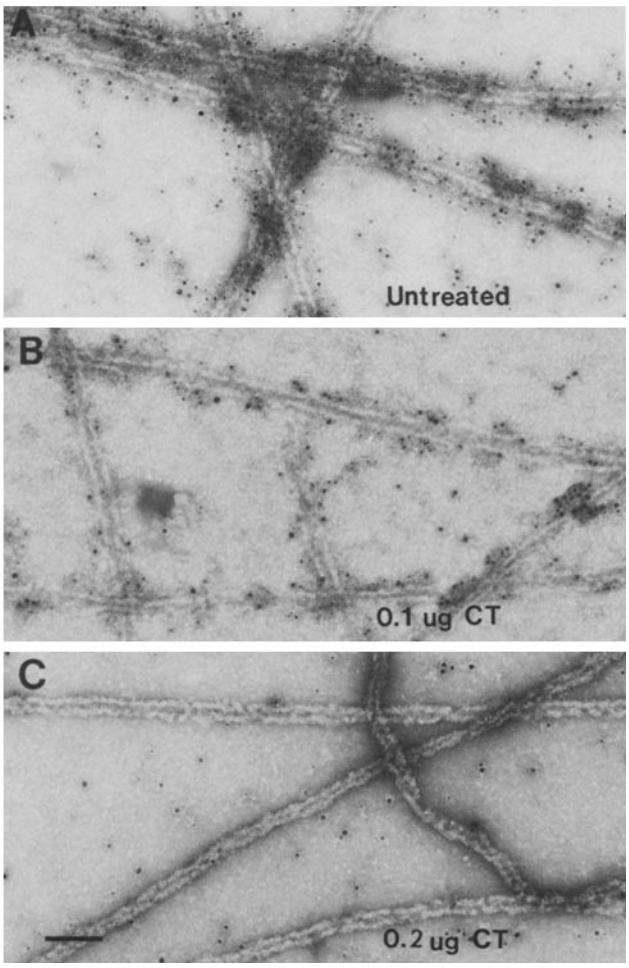

FIGURE 3 Electron micrographs of microtubules treated with alpha-chymotrypsin before labeling with MAP-2 antibody and protein A-gold. Taxol-stabilized microtubules were treated with enzyme for 15 min, arrested with 5 mM phenylmethylsulfonyl fluoride, pelleted through sucrose, resuspended in assembly buffer with glutaraldehyde, and prepared for immunoelectron microscopy as described in Materials and Methods. Microtubules shown in *A* were not treated with enzyme. In *B*, microtubules were treated with 0.1 μg alpha-chymotrypsin/mg MTP as described in Materials and Methods. In *C*, microtubules were treated with 0.2 μg enzyme/mg MTP. ×104,000.

erization at 30°C. Stub antibody also did not depolymerize pre-assembled microtubules over time intervals of 5 to 60 min. These observations indicated that the stub region of MAP-2 must be accessible in order to observe antibody inhibition of assembly.

Comparisons of the Chymotryptic Cleavage Patterns of MAPs On and Off Microtubules

If the stub region was inaccessible to antibody while bound to microtubules, we reasoned that it might also show differential sensitivity to proteolytic enzymes such as alpha-chymotrypsin, depending on whether it was bound to microtubules or free in solution. To investigate this possibility, we compared the rates of appearance of the stub peptides in two samples of taxol-stabilized microtubules, one of which contained 0.3 M NaCl to release MAPs from the microtubules.

TABLE I. *Inhibition of Microtubule Elongation by MAP Antibodies*

Experiment	Preimmune IgG	Percent inhibition* MAP antibody	Arm antibody	Stub antibody
A	0	23	—	40
B	0	<0‡	11	41

In experiment A, the MAP:antibody molar ratio was 10:1. In experiment B the ratio was 1:1. In both series, 700-μl aliquots of high-speed supernate of microtubule protein at 1 mg/ml were incubated with antibody on ice for 15 min. Samples were placed in a cuvette, allowed to equilibrate to 30°C for 45 s, then seeds (25 μl of cycled, polymerized microtubules at 5 mg/ml) were added to the sample in the cuvette. Changes in the turbidity were followed at 350 nm and rates of polymerization were determined from the initial slope according to the methods of Murphy et al. (1977).

*The values represent percent inhibition relative to the preimmune IgG control which is designated 0% inhibition. In fact, the presence of IgG slightly inhibits elongation, the amounts observed relative to PBS controls being 2% for series *A* and 33% for series *B*.

‡Increased turbidity and electron microscopic inspection of the sample indicated immune complexes containing cross-linked microtubules.

Aliquots of both samples were removed after addition of chymotrypsin and examined by immunoblotting using MAP antibody and ^{125}I-protein A to specifically visualize the 36- and 40-kD stub peptides of MAP-2. In control experiments we determined that the MAPs in this sample were free in solution and fully accessible to enzyme and that the rate and extent of digestion of purified MAPs free in solution were identical with or without the addition of salt. In particular, the presence of salt did not alter the rates of appearance and disappearance of the 36- and 40-kD peptides in preparations of purified MAP-2.

In the presence of microtubules, however, differences were observed in the MAP digestion patterns with respect to the stub peptides. In microtubule preparations containing MAPs, both the 36- and 40-kD fragments were generated rapidly but the 36-kD fragment eventually disappeared while the amounts of the 40-kD fragment remained nearly unchanged for at least 30 min (Fig. 4*A*). In preparations containing dissociated MAPs, the 40-kD fragment appeared first and was the predominant fragment as the amounts of the 36-kD fragment gradually increased. After ~10 min, both species began to decline with the 40-kD species disappearing completely by 30 min (Fig. 4*B*). These observations reveal significant differences in the extent and time of appearance of the stub peptides, depending on their state of association with microtubules. In particular, the 40-kD stub peptide appears to be relatively stable to digestion when MAPs are associated with microtubules, suggesting that the cleavage sites responsible for generating this fragment are closely associated with, or partially buried in, the microtubule surface.

Platinum Shadowing of MAP-2 Stub Antibody Complexes

To determine the location and size of the microtubule-binding domain of MAP-2, we incubated intact purified MAP-2 in solution with stub antibody and visualized the antigen–antibody complexes by platinum shadowing. We observed thin, extended molecules similar to those described by Voter and Erickson (1982), but dissimilar to the globular particles described by Zingsheim et al. (1979). Shown in Fig. 5 are representative MAPs with associated stub antibody. The mean length and maximum length of isolated MAPs were 90

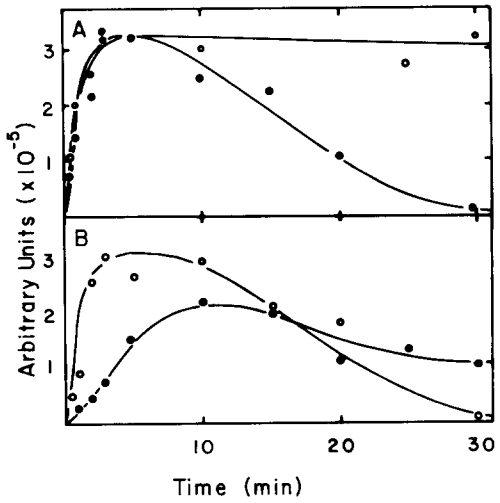

FIGURE 4 Differential chymotryptic cleavage of MAP-2 while bound to microtubules or free in solution. Microtubules stabilized in 10 μM taxol and containing MAPs were digested for various times with alpha-chymotrypsin (0.1 μg chymotrypsin/mg MTP, 37°C). Cleavage products derived from MAP-2 were visualized by immunoblotting using an antibody specific for MAP-2 and ^{125}I-protein A. The figure shows the amount of the 36-kD (●) and 40-kD (○) stub peptides as determined by densitometry of the autoradiogram. Electron microscopic examination confirmed that the microtubules persist throughout the digestion period. (*A*) Digestion of microtubules with associated MAPs in assembly buffer. (*B*) Digestion of microtubules in assembly buffer with 0.3 M KCl to release MAPs from microtubule surfaces.

± 30 nm and 125 nm, respectively, but in one or two cases MAPs 170 nm long were observed (Fig. 6). These values are somewhat shorter than the value of 185 nm reported by Voter and Erickson (1982). The large variability in length (SD = 30 nm) suggests that the conformation of isolated MAPs may be variable; although gel electrophoresis showed that some proteolysis of MAP-2 had occurred, the extent of proteolysis is probably too minor to account for the variability in the lengths we observed. The width of MAP-2 is probably less than the actual measured value of 40 nm, since the isolated MAPs could only be detected after unidirectional shadowing at a low angle (6°) and completely disappeared after rotary shadowing.

In surveying the platinum-shadowed samples, both free MAPs and MAP–antibody complexes could be found. As shown in Fig. 7, the striking feature was that >90% of the complexes had antibody bound near one end and within one-quarter of the extended MAP-2 molecule; the remaining 10% had antibody attached in the middle one-third of the protein. However, in these cases the MAPs were found to be much longer than the average length, suggesting that the bifunctional antibody had bound two MAPs end-to-end. In no case was antibody observed bound to both ends of the molecule. If these unusual complexes are eliminated, we conclude that the microtubule-binding domain is located at just one end of the molecule. We estimate that the position of the 35–40-kD region recognized by stub antibody is located within 40 nm of one end of the MAP terminus. The stub domain may therefore comprise up to one-third of the length of an extended molecule with a length of 125 nm.

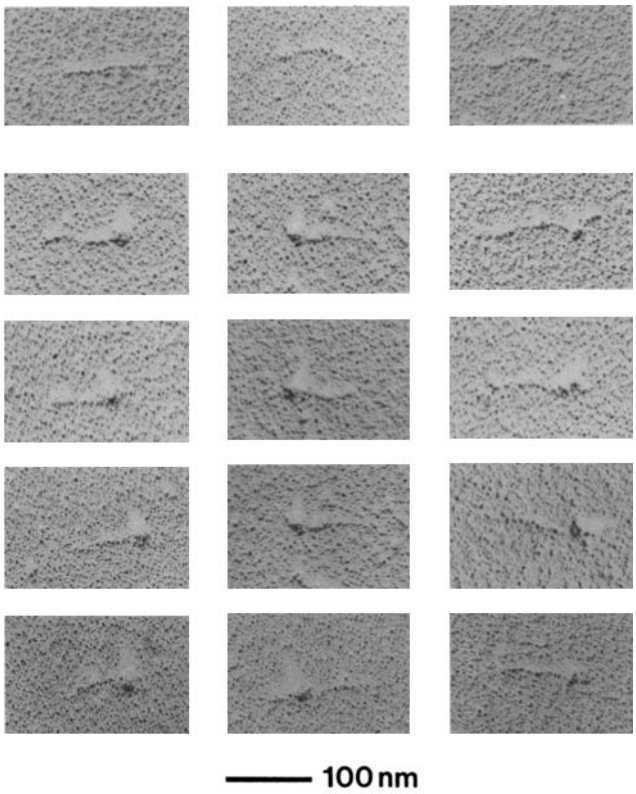

FIGURE 5 Platinum shadowing of isolated MAP-2 after incubation with MAP stub antibody. MAPs were incubated with equimolar amounts of stub antibody and diluted in 20% glycerol as described in Materials and Methods, sprayed onto mica with an atomizer, and unidirectionally shadowed with platinum at an angle of 6°. The antibody is located at one end of the extended MAP molecule. The top row shows MAP molecules that are not labeled with antibody. Bar, 100 nm. ×130,000.

DISCUSSION

MAP-2 Contains a Single Antigenic Determinant to the Microtubule-binding Domain

Using affinity chromatography, we subfractionated rabbit antibodies to whole MAP-2 into two fractions that are specific for the respective "stub" and "arm" domains of the molecule. Stub antibody is quite specific after passage over an arm column and its specificity is not significantly improved by double-column treatment (arm column followed by stub column). The fact that stub antibody also labels high molecular weight fragments suggests that large immunoreactive fragments containing the stub region are also generated by the chymotryptic cleavage procedure. This is consistent with the observations of Scherson et al. (1982), who described an alternative cleavage pathway in which chymotrypsin cleaves the stub region and much of the projecting portion of MAP-2 and suggests that a certain percentage of MAPs are free in solution. We do not feel that the binding of stub antibody to high molecular weight peptides represents common antigenic determinants between stub and arm, because double-column purification would eliminate all cross-reactivity, rather than give rise to the results we observe, i.e., no change in the labeling pattern by stub antibody and a significant reduction in labeling of the 35–40-kD stub by arm antibody. Our observations of platinum shadowing of stub antibody–MAP-

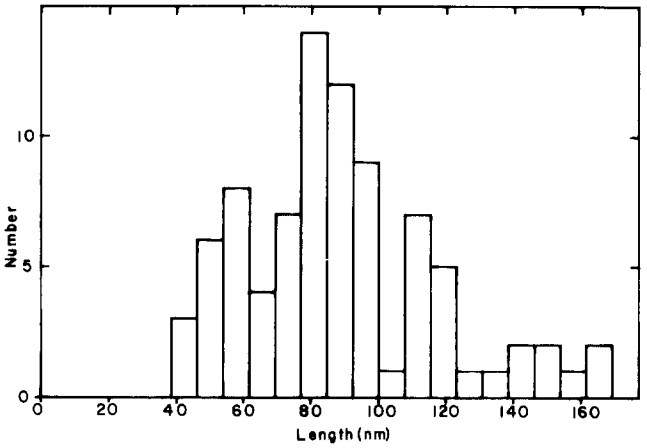

FIGURE 6 Length distribution of MAP-2. Measurements were made from enlarged photographs of platinum-shadowed MAP–antibody complexes (n = 93). The mean length was 90 ± 30 nm. The maximum length observed was 170 nm.

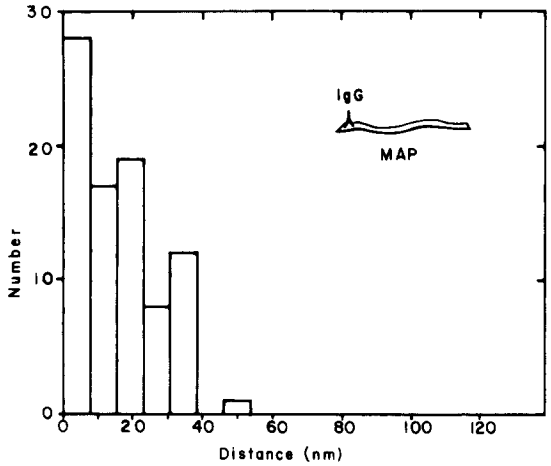

FIGURE 7 Determination of the size and position of the stub region on isolated MAP molecules. The measurements indicate the space between the antibody and the nearest end of the molecule. Measurements were made from photographic enlargements to nearest whole millimeter, where the average maximum MAP length was 16 mm (125 nm). Stub antibody bound most frequently at a position ranging from 0 to 40 nm from one end of the molecule.

2 complexes also argue against the possibility of multiple antigenic determinants within the molecule, since we consistently observed antibody binding to only one end of the molecule.

The Microtubule-binding Domain May Become Hidden When MAPs Are Associated with Microtubules

The stub antibody we prepared did not label microtubules and was therefore not useful for examining the periodic distribution of MAP-2 on microtubules. It is possible that the antigenic sites on the stub are inaccessible to antibody when associated with the microtubule wall, which could happen if the stub region of MAP-2 were located in a groove between adjacent tubulin subunits, wide enough to accommodate the thin MAP filament (reported by Voter and Erickson [1982] to be >1.5 nm), but inaccessible to antibodies. Similarly, our analysis of the chymotryptic digestion products of MAP-2 indicated differential cleavage at sites resulting in the 36- and 40-kD stub peptides, with the 40-kD peptide being generated more rapidly for dissociated MAPs and being relatively more stable for microtubule-associated MAPs. Thus chymotrypsin may also have limited access to MAPs bound to the microtubule wall. That the stub region is inaccessible to antibody while bound to microtubules is also supported by our observations on the effects of stub antibody on microtubule assembly and stability (see below). An alternate idea that has not yet been ruled out is that the MAP stub remains exposed and that the antigenic sites are hidden or undergo conformational changes upon microtubule binding, but we do not consider these likely, given the consistency of antibody and chymotrypsin experiments and the polyclonal nature of the rabbit antibody to stub.

MAP Stub Antibody Inhibits Microtubule Assembly

Incubation of MAP or stub antibody with ring-free microtubule protein interferes with subsequent elongation onto added seeds. To observe the effect it was necessary to preincubate the antibody with ring-free preparations of soluble microtubule protein at 5°C prior to assembly at 30°C. We were unable to induce depolymerization of assembled microtubules, presumably because the region of MAP-2 necessary for microtubule stabilization is inaccessible to antibody. We also found that the presence of rings prevented the inhibitory effects of stub antibody on microtubule elongation, suggesting that the stub region of MAP-2 is also protected in tubulin ring oligomers, and we conclude that during microtubule elongation, the stub region may be accessible for only a brief time or not at all. Although our own preliminary evidence suggests that MAPs exchange on and off microtubules, so that they should be accessible to stub antibody at least some of the time, MAP antibody did not promote the disassembly of preformed microtubules; however, we were restricted to low concentrations of antibody, and this may have limited our ability to see an effect.

The manner in which MAPs stabilize microtubules and the role they may play in nucleation, formation of rings, and assembly of microtubules are not well understood, and further work is needed. However, these studies provide preliminary evidence that a portion of the microtubule binding site of MAP-2 may be hidden when MAPs are bound to microtubules or to tubulin oligomers. Since different regions of the MAP-2 molecule may be involved in different functions, the use of stub and arm antibodies may provide new information about the way in which MAPs interact with tubulin and other cytoskeletal components.

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