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Mapping of Distinct Structural Domains on Microtubule-Associated Protein 2 by Monoclonal Antibodies

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Monoclonal antibodes against microtubule-associated protein 2 (MAP2) were prepared and their specificity was verified by visualization of the antigens using the antibody overlay technique and by radioimmunoassay. MAP2 was cleaved by α-chymotrypsin to generate a series of high-molecular-mass fragments ranging between 270 and 140 kDa. The precursor-product relationship of these fragments was suggested from the rate of their appearance and from the analysis of the tryptic peptide map of each fragment. A group of monoclonal antibodies was found to react predominantly with the intact 270-kDa MAP2 molecule and a fragment having a mass of 240 kDa and to some extent with a 215-kDa fragment. Another group of monoclonal antibodies reacted with an antigenic determinant which was located on the 270-kDa molecule as well as on fragments as small as 140 kDa. None of the two groups of monoclonal antibodies reacted with the microtubule-binding domain of MAP2. These results suggest that one group of antibodies reacts with sites located at or dependent upon a terminal 60-kDa domain(s) distal to the microtubule-binding site of MAP2. The second group of antibodies, which can still bind to smaller proteolytic products, appear to be associated with the central region of the MAP2 molecule. Indirect immunofluorescence experiments with the antibody preparations indicated that at least some of the antigenic determinants are exposed when MAP2 is associated with microtubules in the cell body and neurite outgrowths of differentiated rat brain neuroblastoma B104 cells.

Microtubules, isolated by successive cycles of assembly-disassembly, are composed of tubulin and several additional proteins designated microtubule-associated proteins or MAPs. Two major groups of MAPs have been identified in neutral tissues and in cultured cells. These include high-molecular-mass components (350 and 270 kDa) termed MAP1 and MAP2, respectively [1 – 5] and a number of polypeptides in the molecular mass range of 55 – 68 kDa, known collectively as τ proteins [6 – 8]. Both MAP2 and τ factors have been shown to promote nucleation and elongation of microtubules *in vitro* [1 – 7, 9, 10], but their role in the control of microtubule assembly and its interactions in the living cells is still uncertain.

The most prominent MAP components in neuronal tissue are the high-molecular-mass proteins MAP1 and MAP2. The latter was found by electron microscopy to associate with microtubules at a fixed periodicity forming laterally projected arms [2, 11, 12]. The tissue and species distribution of MAP2 is still controversial at the present time. Immunocytochemical data suggest that this protein is associated with microtubules from a wide variety of cells and tissues [13 – 17]. Other groups, on the other hand, have used multispecific and monoclonal antibodies to show that MAP2 is present only in cells of neuronal origin [18], while other cells, such as HeLa, contain distinct types of MAP components with molecular masses of 125 kDa and 210 kDa [19, 20].

Several studies point to additional activities of MAP2, beside its capacity to bind to microtubules. It has been shown that MAP2 may induce actin polymerization *in vitro* [21] and that it is associated with a protein kinase activity [22]. These data, together with the indications that microtubules may be associated with other cytoskeletal networks and with cellular organelles [23 – 27], suggest the possibility that MAP2 may be involved in mediating such interactions. Thus, it is possible that the side arm of MAP2 which is projected away from the microtubules contains functionally important domains.

To probe the various regions of the MAP2 molecule we have prepared monoclonal antibodies to this molecule. We report here on the mapping of distinct structural domains on MAP2 by monoclonal antibodies and use of these antibodies for the localization of the MAP2 molecule in cultures of cells of neuronal origin.

MATERIALS AND METHODS

Antigen Preparation

Microtubule proteins were prepared from calf brains by two cycles of assembly-disassembly [28] and stored as pellets at −70 °C. The MAPs used for immunization were separated from tubulin by phosphocellulose chromatography [8]. For the screening of hybridoma antibodies phosphocellulose-purified tubulin was used. MAP2 and τ proteins used for the screening assays were isolated by thermal denaturation of microtubule proteins and further purified on an Ultrogel AcA 34 column [7].

Abbreviations. MAP1 and MAP2, microtubule-associated proteins 1 and 2; Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonic fluoride; P_i/NaCl, phosphate-buffered saline; Bt_2cAMP, 1,N^6-dibutyryl-adenosine 3′,5′-monophosphate; Pipes, 1,4-piperazinesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC-tubulin, tubulin isolated by phosphocellulose chromatography.

Monoclonal Antibody Preparation

Balb/c female mice were immunized at the age of 6–8 weeks. The antigen, 50 μg/mouse, was injected into the foot pad in complete Freund's adjuvant. After 21 days a booster injection (50 μg/mouse) was applied intraperitoneally and four days before fusion the antigen was applied again intravenously. The mice were then sacrificed and the spleens excised. Cells, 1.3×10^8, were fused with 1×10^7 logarithmic phase × 63 NS1/1-AG-4 (NSI) myeloma cells in 40% (w/v) poly(ethylene glycol) 1500. Fused cells were grown in Dubecco's minimum essential medium supplemented with heat-inactivated 10% horse serum, 1 μM hypoxanthine, 4 nM aminopterine and 0.16 μM thymidine. Fourteen days after fusion cell culture supernatants were screened for the production of specific antibodies by solid-phase radioimmunoassay [29]. Positive clones were subcloned in Dubecco's minimum essential medium containing 20% heat-inactivated horse serum and 0.5% agar. Selected clones were injected into Pristane primed mice and ascites fluid collected.

Assay of MAP2 Antibody Specificity

In order to determine the specificity of the MAP2 antibodies, proteolytic fragments of the MAP2 molecule were prepared from either purified MAP2 or by digestion of microtubules under conditions in which tubulin was not degraded [30]. To produce fragments from purified MAP2 molecules, 20 μg of MAP2 in 25 μl of Mes buffer (0.3 M Mes, pH 6.6; 1 mM EDTA; 1 mM $MgCl_2$; 2 mM dithiothreitol) were incubated with 3 μg of chymotrypsin (Worthington Biochemical Corp.) at 37 °C for 8 min. The reaction was stopped by addition of electrophoresis sample buffer (5% 2-mercaptoethanol, 10% glycerol, 2.3% w/v SDS, 0.062 M Tris pH 6.8). For the preparation of MAP2 fragments from microtubules, 1 mg of calf brain microtubules prepared by three cycles of assembly-disassembly were resuspended in 200 μl of Mes buffer and incubated at 37 °C with 1 mM GTP in the absence of glycerol. After 10 min, 76 ng of α-chymotrypsin were added and the mixture was further incubated for 8 min and 16 min respectively at 37 °C. The reaction was stopped with 2 mM phenylmethylsulphonyl fluoride (PMSF). The microtubules were sedimented at 102000 × *g* for 30 min. The supernatant was collected and the microtubule pellet (depleted of the MAP2 projecting 'arms') resuspended in Mes buffer. This microtubule suspension was depolymerized for 30 min at 4 °C and the aggregates removed by centrifugation at 186 kPa for 10 min in a Beckman airfuge. For solid-phase radioimmunoassay, 25 μl of either the microtubule suspension (5 mg/ml) or the supernatant fraction (0.5 mg/ml) were adsorbed onto the polyvinyl microplates (Dynatech) at room temperature for 4 h. The wells were washed three times with P_i/NaCl containing 1% bovine serum albumin. The monoclonal antibodies (25 μl) were then added and incubation continued for 4 h. The wells were washed three times with the p_i/NaCl/albumin and 25 μl of ^{125}I-labelled goat anti-mouse antibodies (10^5 counts/min) were added and inucbated overnight at 4 °C.

Antibody Staining of Proteins Resolved by Electrophoresis on SDS/Polyacrylamide Gels

Tubulin, MAP2 or proteolytic fragments of MAP2 were subjected to SDS/8% polyacrylamide gel electrophoresis and visualized by the antibody overlay technique [31]. Following electrophoresis the gels were fixed for 2 h in 20% methanol containing 7% acetic acid, followed by several washings (8–10 h) with 10 mM Tris, 150 mM NaCl, 0.1% NaN_3, pH 7.2 (Tris/NaCl/NaN_3 buffer). The gel was cut longitudinally into 1-cm strips which were incubated overnight with various monoclonal antibodies diluted in Tris/NaCl/NaN_3 buffer containing 10% normal goat serum in 15-ml stoppered plastic conical culture tubes with continuous shaking. After extensive washings with Tris/NaCl/NaN_3 buffer the gel strips were incubated with ^{125}I-labelled goat anti-mouse antibodies (2×10^6 counts/min) in Tris/NaCl/NaN_3 buffer containing 10% normal goat serum. The strips were extensively rinsed with Tris/NaCl/NaN_3 buffer, dryed and exposed to X-ray film. Densitometric scanning of the autoradiograms was carried out in a Gilford spectrophotometer at 595 nm.

Two-Dimensional Peptide Map Analysis

Purified MAP2 was digested with α-chymotrypsin as described above. The proteolytic products were subjected to electrophoresis in an SDS/8% polyacrylamide slab gel [32]. The gel was stained with 0.2% Coomassie brilliant blue in 20% methanol, 7% acetic acid, and destained with 20% methanol, 7% acetic acid. The bands corresponding to each MAP2 fragment were excised from the gel, washed with 10% methanol for 4 h and lyophylized. The dried gels were placed in siliconized tubes to which 22 μl of 0.5 M sodium phosphate buffer, pH 7.5; 3 μl of [^{125}I]iodine (Amersham 100 mCi/ml) and 5 μl of chloramine T (1 mg/ml) were added. The mixture was incubated with the gel slices for 30 min. The reaction was terminated by addition of 1 ml (1 mg/ml) of sodium bisulfite, and the mixture incubated for 5–15 min at room temperature. The solution was removed and the tubes filled with 10% methanol. The gels were washed twice a day with 10% methanol for 3 days. The methanol was then removed and the samples lyophilized. A solution (1 ml) containing 50 μg of trypsin (treated with L-1-tosylamido-2-phenylothyl chloromethyl ketone) in 0.05 M NH_4HCO_3, pH 8.0 was added to each tube. The protein fragments in the gels were allowed to undergo proteolysis for 25 h at 37 °C. The samples were lyophilized again. To the tubes 30 μl of electrophoresis buffer (acetic acid/formic acid/water; 15 : 5 : 80) were then added and 1–3 μl of the sample were spotted onto a thin-layer cellulose-coated chromatography plate (Merck). Each sample was applied in the cathode corner and subjected to electrophoresis at 1000 V and the plate dried at 80 °C. For the second dimension each plate was chromatographed in butanol/pyridine/acetic acid/water (32.5 : 25 : 5 : 20) and then dried. Autoradiograms were obtained by exposing the plates to Kodak Blue Band X-ray film and Dupont intensifying screens for 15–24 h at −70 °C [33].

Immunofluorescence

Rat brain neuroblastoma B104 cell line, isolated from a nitrosethylurea-induced neoplasm [34,35] was kindly provided by D. Schubert of the Salk Institute (La Jolla, CA). Cultures were grown to the confluent state in Dubecco's minimal essential medium supplemented with 8% fetal calf serum in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The cells were plated on polylysine-coated (5μg/ml) coverslips and induced to differentiate in Dubecco's minimal essential medium supplemented with 10% fetal calf serum and 1 mM Bt_2cAMP for four days at 37 °C. The coverslips were washed three times with Pipes/Hepes/$MgCl_2$/EGTA buffer (60 mM Pipes, 25 mM Hepes, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) and the cells

were lysed in this buffer containing 0.2 % Brij 58 for 5 min at room temperature [36]. The supernatant was removed and the extracted cells were then fixed with 0.5 % glutaraldehyde in Pipes/Hepes/$MgCl_2$/EGTA buffer for 15 min. The coverslips were washed three times with this buffer and the cells treated three times with 1 mg/ml $NaBH_4$ in Pipes/Hepes/$MgCl_2$/EGTA buffer, for 5 min each at room temperature, followed by several buffer washings. The coverslips were then inverted onto a 50-µl drop of the corresponding affinity purified antibody solution (0.015 mg/ml in P_i/NaCl) and incubated for 30 min. The coverslips were washed three times with P_i/NaCl and inverted again onto 50-µl drops of rhodamine-conjugated goat anti-rabbit or anti-mouse immunoglobulin. The coverslips were mounted on glass slides with 90 % glycerol in P_i/NaCl and viewed in a Zeiss Photomicroscope III equipped with filters for fluorescein and rhodamine fluorescence.

RESULTS

Preparation of Monoclonal Antibodies Against MAP2

Calf brain microtubules were purified by two cycles of assembly and disassembly and fractionated by phosphocellulose chromatography to separate the tubulin (PC-tubulin) from the MAPs. For MAP isolation the microtubule fraction was heated and the high-molecular-mass MAP2 was then separated from the τ proteins by Ultrogel chromatography. The results shown in Fig. 1 indicate a complete separation of the three groups of proteins. For immunization of mice we injected the entire MAP fraction (MAP2 and τ) though the screening of antibody-producing clones was performed with the purified components. We have often detected limited proteolysis of MAP2 upon electrophoresis of overloaded SDS/polyacrylamide gels. This observation will be further discussed below.

From the hybridoma cultures producing antibodies against MAP2 eight positive clones which were found to be IgG producers were selected. We have also obtained monoclonal antibodies against tubulin which reacted with this protein specifically and not with MAP2 or τ. The specificity of each antibody preparation was verified by solid-phase radioimmunoassay, as well as by radioimmunolabeling of gels following electrophoretic separation of the proteins (or their blotted replicas). The example shown in Fig. 2 indicates that monoclonal antibodies against MAP2 bind to this protein specifically while monoclonal antibodies against tubulin associate only with the respective antigens.

Interaction of the Various Monoclonal Antibodies with Proteolytic Fragments of MAP2

As mentioned above, purified MAP2 preparations often display several additional high-molecular-mass bands which are the products of limited endogenous proteolysis [4]. Upon storage of MAP2 solutions these fragments become more prominent, accompanied by a parallel decrease in the level of intact MAP2. Radioimmunolabeling of electrophoretic gels with the various monoclonal antibodies indicated that two clones produced antibodies which bind predominantly in a broad band corresponding to the intact molecule and to a 240-kDa fragment; a limited reactivity was also observed with a 215-kDa fragment. Another group of antibodies consisting of six independent clones also reacted efficiently with smaller fragments with apparent molecular masses of 180 kDa and 140 kDa. Examples showing densitometric scanning of the representative autoradiographs are shown in Fig. 3. It should be emphasized that the MAP2 solution applied for electrophoresis was identical in both cases. These results suggested that different monoclonal antibodies recognize distinct regions along the MAP2 molecule.

To characterize further the different antigenic 'domains' of MAP2, we subjected the protein to the controlled α-chymo-

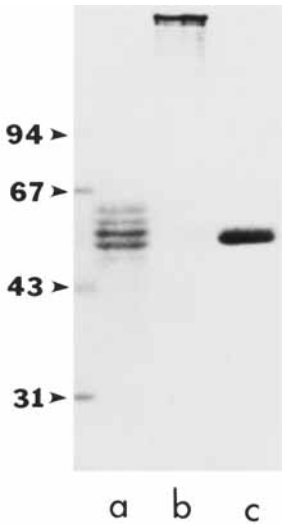

Fig. 1. *SDS/polyacrylamide gel electrophoresis of purified τ, MAP2 and tubulin.* Microtubules were isolated from calf brain by two cycles of assembly-disassembly. Tubulin was purified by phosphocellulose chromatography. MAPs were purified from heat-inactivated microtubules followed by gel filtration to separate τ from MAP2. (a) τ; (b) MAP2; (c) tubulin. The arrows indicate molecular masses of markers in kDa (phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 31)

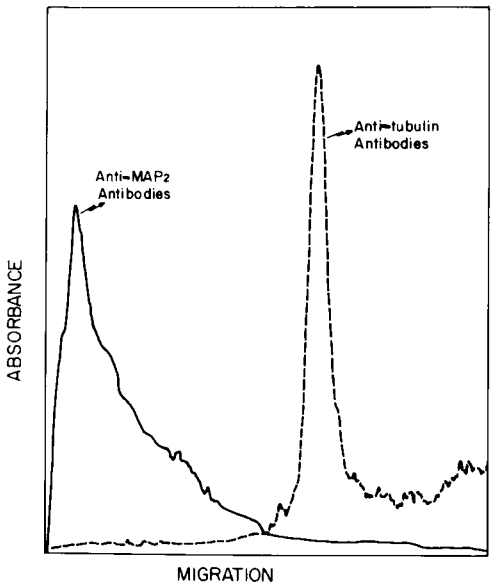

Fig. 2. *Antigenic specificity of monoclonal antibodies against MAP2 or against tubulin.* Purified MAP2 and tubulin were prepared as described under Fig. 1. 3 µg of each protein solution were mixed together and subjected to electrophoresis in an SDS/8 % polyacrylamide slab gel. Parallel gel strips were overlayed with either monoclonal antibodies against MAP2 or tubulin, followed by ^{125}I-labelled goat anti-mouse immunoglobulin (see Materials and Methods)

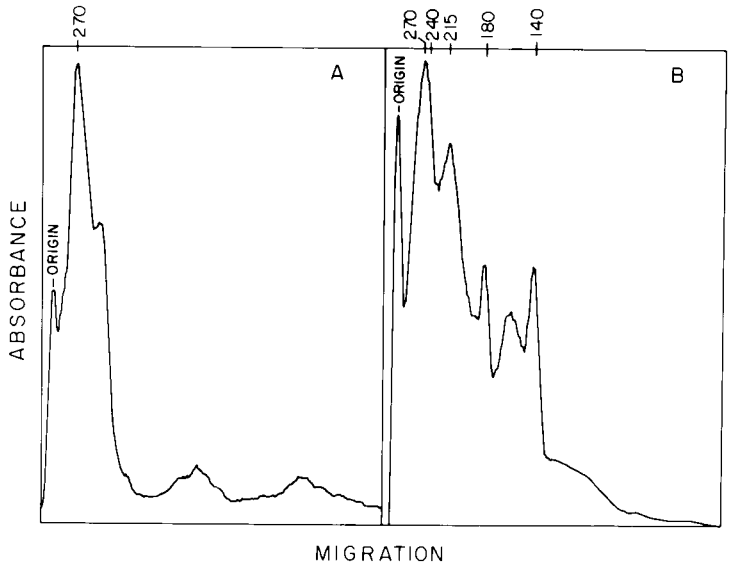

Fig. 3. *Radioimmunolabeling of MAP2 and its endogenous proteolytic fragments with two antibodies against MAP2 displaying different specificities.* To each slot of an SDS 8 % polyacrylamide slab gel, 10 µg of purified MAP2 solution was added. Following electrophoresis the gel was cut into longitudinal strips which were visualized by the overlay technique using different monoclonal anti-MAP2 antibodies as described under Fig. 2 and in Materials and Methods. (A) Densitometric scanning of the specificity displayed by group A monoclonal antibodies. These antibodies bind to intact MAP2 and 240-kDa and 215-kDa fragments. (B) Group B monoclonal antibodies which bind to MAP2, 240-kDa, 215-kDa, 180-kDa and 140-kDa fragments. Numbers at the top indicate molecular masses in kDa

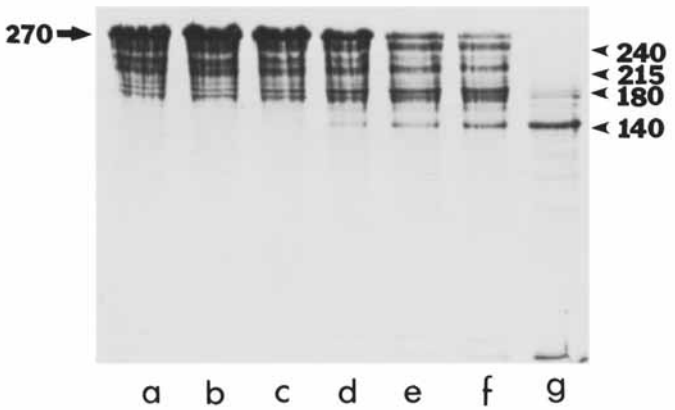

Fig. 4. *SDS/polyacrylamide gel electrophoresis of α-chymotrypsin digestion products of MAP2.* The reaction mixture in 25 µl of Mes buffer, contained 20 µg of purified MAP2 and increasing amounts of α-chymotrypsin. The mixtures were incubated 8 min at 37 C and subjected to SDS/polyacrylamide gel electrophoresis. (a) No enzyme; (b) 0.5 ng; (c) 1 ng; (d) 2 ng; (e) 5 ng; (f) 8 ng; and (g) 10 ng of α-chymotrypsin. The numbers refer to molecular masses in kDa

trypsin digestion according to the procedure of Vallee [30]. The results of digestion of MAP2 by increasing concentrations of α-chymotrypsin (1 – 10 ng α-chymotrypsin, 20 µg of MAP2 in 25 µl) are shown in Fig. 4. The α-chymotrypsin treatment resulted in the formation of several prominent fragments with apparent molecular masses of 240, 215, 180, and 140 kDa (Fig. 4). The incubation with the highest amount of α-chymotrypsin (10 ng) resulted in complete digestion of MAP2 and the appearance of cleavage products of lower molecular mass. It should be noted that the amounts of MAP2 applied to the gel were very high (20 µg in lane a, Fig. 4; as compared to 4 µg in Fig. 1, lane b).

In order to characterize further the different α-chymotryptic peptides, the individual bands were excised from the stained gel, iodinated, cleaved by trypsin and analyzed by two-dimensional electrophoresis and chromatography. The autoradiograms of the tryptic maps obtained from MAP2 and the 240-kDa, 215-kDa, 180-kDa, and 140-kDa fragments are depicted in Fig. 5. The gradual disappearance of radioactive spots (some of which/are indicated by bars in Fig. 5) suggests that the fragments with molecular masses of 240, 180, and 140 kDa may be derived by sequential proteolysis. The 215-kDa component was an exception in that it displayed at least one major ^{125}I-labelled peptide which is hardly detected in the larger 240-kDa fragment. The 215-kDa peptide may therefore be derived by an alternative cleavage pathway directly from the intact MAP2 molecule.

Analysis of the two groups of monoclonal antibodies described above with respect to their interaction with the defined chymotryptic peptides is shown in Fig. 6. Again two populations of antibodies were resolved: those reacting predominantly with the intact MAP2 molecule, and to some extent with the 240-kDa and the 215-kDa peptides (Fig. 6A), and those which bind efficiently to the smaller fragments as well (Fig. 6B). When the same electrophoretic gels were incubated with irrelevant IgG-producing monoclonal antibodies, no binding was obtained (Fig. 6C).

The Relationship between Defined Antigenic Domains on MAP2 and Its Site of Interaction with Microtubules

We have examined whether the portion of MAP2 molecule which is recognized by each of our monoclonal antibodies is within or proximal to the microtubule-binding site of MAP2. The monoclonal antibodies were mixed with MAP2, centrifuged to remove any aggregates formed and then added to PC-tubulin. We first ascertained that the antibodies do not cause precipitation of the MAP2 molecules. Analysis of the extent of tubulin polymerization indicated that the different antibodies did not prevent the assembly process nor did they affect the rate of polymerization. Moreover, the antibody treatment (in

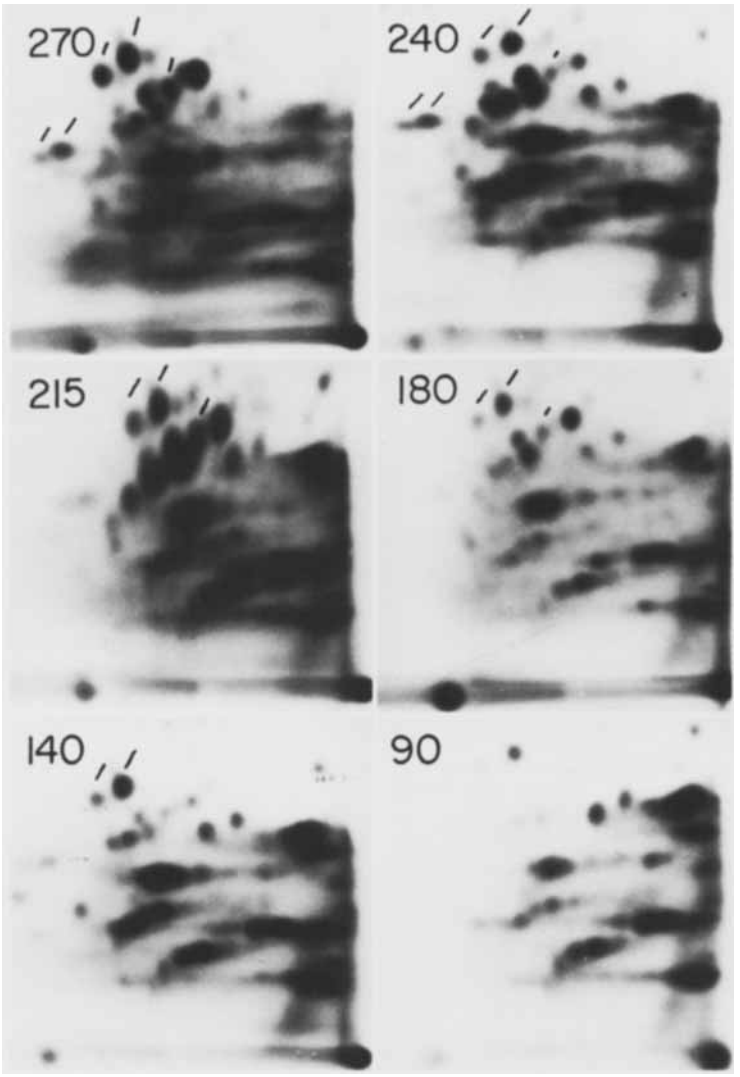

Fig. 5. *Two-dimensional peptide maps of MAP2 and its α-chymotryptic fragments.* Purified MAP2 was digested with 5 ng of α-chymotrypsin as described under Fig. 4(e). Following gel electrophoresis the gels were stained with Coomassie brilliant blue as depicted in Fig. 4(e). The α-chymotryptic fragments were labeled with ^{125}I and digested with trypsin as described in Materials and Methods. The numbers in each insert indicate the molecular mass in kDa of the initial α-chymotryptic fragments. The bars indicate some of the larger common cleavage peptides

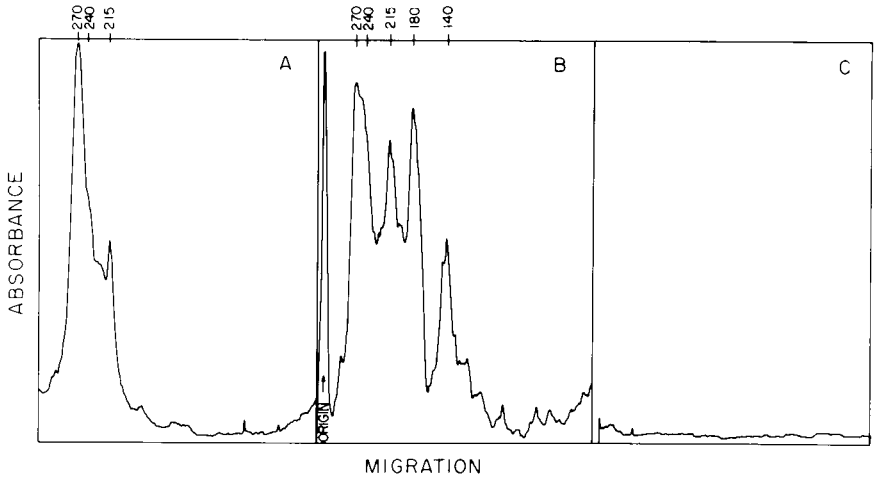

Fig. 6. *Radioimmunolabeling of α-chymotrypsin digestion products of MAP2 monoclonal antibodies against MAP 2 antibodies displaying different specificities.* Reaction mixtures in 25 µl of Mes buffer contained 20 µg of purified MAP2 and 3 ng of α-chymotrypsin. Following incubation for 8 min at 37 C the mixtures were subjected to SDS/polyacrylamide slab gel electrophoresis. The gel was cut into longitudinal strips which were visualized by the overly technique using different monoclonal antibodies against MAP2 as antibodies as described under Fig. 2 and in Materials and Methods. (A) Densitometric scanning of the specificity displayed by group A monoclonal antibodies. These antibodies bind to intact MAP2, 240-kDa and 215-kDa fragments. (B) Group B monoclonal antibodies which bind to MAP2, 240-kDa, 215-kDa, 180-kDa and 140-kDa fragments. (C) Monoclonal antibodies isolated from the same fusion experiments which were unreactive against MAP2. Numbers at the top represent molecular masses in kDa

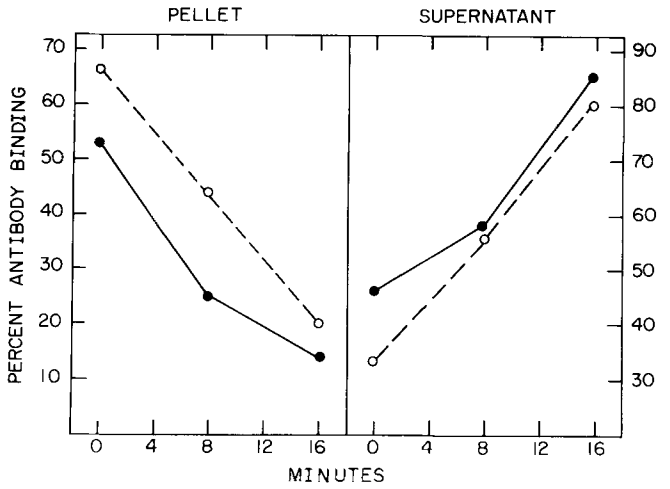

Fig. 7. *Relationship between the microtubule-binding sites on MAP2 and the determinants recognized by the monoclonal antibodies.* Microtubules (1 mg) purified by three cycles of assembly-disassembly were resuspended in 200 µl of Mes buffer. The microtubule suspension was incubated 10 min at 37 °C in the presence of 1 mM GTP. α-Chymotrypsin (76 ng) was added and the mixture was further incubated for 8 min and 16 min respectively at 37 °C. The reaction was stopped by addition of PMSF to a final concentration of 2 mM. The microtubules were sedimented at 102000 × *g* for 30 min and the supernatant collected (0.5 mg/ml). The pellet was resuspended in 200 µl of Mes buffer and centrifuged in a Beckman airfuge (5 mg/ml). Both the first supernatant and pellet fraction were subjected to solid-phase radioimmunoassay using 25 µl of each fraction per well of the microtiter plate followed by incubation with the corresponding monoclonal antibodies diluted 1 :1000. The dashed line represent the reaction with group B monoclonal antibody. The solid line represents the reaction with group A antibodies

antibody concentrations of up to 5 mg/ml) did not inhibit the binding of added MAP2 to either assembled microtubules or to PC-tubulin during its assembly (data not shown).

To determine further the relationships between the microtubule-binding sites on MAP2 and the determinants recognized by the monoclonal antibodies, we have exposed MAP2-containing microtubules to α-chymotrypsin and measured by radioimmunoassay the association of the antigenic activity with the insoluble microtubules and those fragments released to the supernatant by the enzymatic treatment. The results depicted in Fig. 7 indicate that, following incubation with α-chymotrypsin, the antigenic determinants recognized by both groups of monoclonal antibodies are released from the microtubular backbone and appear in the supernatant. It should be noted that electrophoretic analysis of the reaction mixture indicated that the enzymatic digestion did not affect the tubulin molecules. The total antigenic activity was not grossly affected by this digestion treatment. Longer exposure to α-chymotrypsin resulted in progressive digestion of the MAP2 molecule with the concomitant loss of antigenicity. Generally the behavior of the two types of monoclonal antibodies was similar, though in some experiments the decrease in the total antigenic activity occurred at an apparently faster rate when assayed with those antibodies specific to the larger cleavage peptides.

Indirect Immunofluorescence Visualization of MAP2 in Cultured Neuronal Cells

Indirect immunofluorescence experiments were used to determine whether the antigenic determinants that react with the antibodies are exposed or masked when MAP2 is associated with microtubules or other cell structures. Cultured rat brain neuroblastoma B104 cells which were induced to differentiate with Bt_2cAMP were permeabilized with Brij 58, fixed with glutaraldehyde and immunolabelled for tubulin, using rabbit anti-(chicken tubulin) antibodies purified by affinity chromatography, and for MAP2 using rabbit anti-(calf MAP2) antibodies and mouse anti-(calf MAP2) monoclonal antibodies, both putified by affinity chromatography. Tubulin-immunolabelling patterns consisted of elaborate networks of wavy filaments throughout the cell body (Fig. 8A) and the elongated processes (Fig. 8B).

Most of the visualized fibers appear in similar directional orientation and extent towards the neurites. However, a few fibers appear to be organized perpendicularly to the rest of the microtubule fibers. The immunolabelling with either rabbit antibodies or with group A monoclonal antibodies to MAP2 (Fig. 8C and 8D) resulted in a similar labelling pattern. The labelling was associated with the microtubular network, though the intensity of labelling was reduced compared to that observed with tubulin antibodies. It should be noted that MAP2 molecules in cultured cells are easily lost upon extraction and adequate fixation should be employed as previously indicated [36].

DISCUSSION

MAP2 is believed to participate in the regulation of microtubule organization (see [1 – 5,9]). Electron microscopic studies with reconstituted microtubules indicated that MAP2 molecules are bound to and radially projected from the microtubular core with a fixed periodicity [2,11,12,30] and that proteolytic digestion of MAP2-containing microtubules results in the specific removal of the projecting arms leaving behind a microtubule-binding peptide of about 32 kDa [30]. Besides the tubulin-binding domain of MAP2 other functions have been assigned to the 'arm' of the molecule. These include cAMP-dependent protein kinase activity associated with that region, the capability to undergo phosphorylation and perhaps the capacity to mediate interaction of the microtubules with other cytoplasmic elements [22 – 27]. It is conceivable that the characterization of specific functional domains in the MAP2 molecule will require defined probes which exhibit restricted specificity towards distinct sites along the molecule.

The approach employed here was the preparation of specific monoclonal antibodies which react with different loci along the MAP2 molecule. The reactivity of the various antibodies with α-chymotryptic cleavage peptides of MAP2 as well as with products of endogenous proteolysis revealed two major groups of monoclonal antibodies: (A) those which react mainly with the intact MAP2, the 240-kDa and 215-kDa fragments; and (B) those which also recognize smaller peptides of 180 kDa and 140 kDa. The precursor-product relationship of these chymotryptic fragments was suggested from their rate of appearance and the analysis of the tryptic peptide map of each fragment. Based on these results and the molecular model of MAP2 proposed by Vallee [30], it is suggested that the 240-kDa, 180-kDa, and 140-kDa peptides are products of sequential digestion by α-chymotrypsin. We may also assign possible locations for the antigenic sites reactive with the monoclonal antibodies. This model [30] assumes that the first point of cleavage is at the hinge region between the 32-kDa microtubule-binding domain and the 240-kDa side arm (arrow 1, Fig. 9). The second chymotryptic cleavage removes

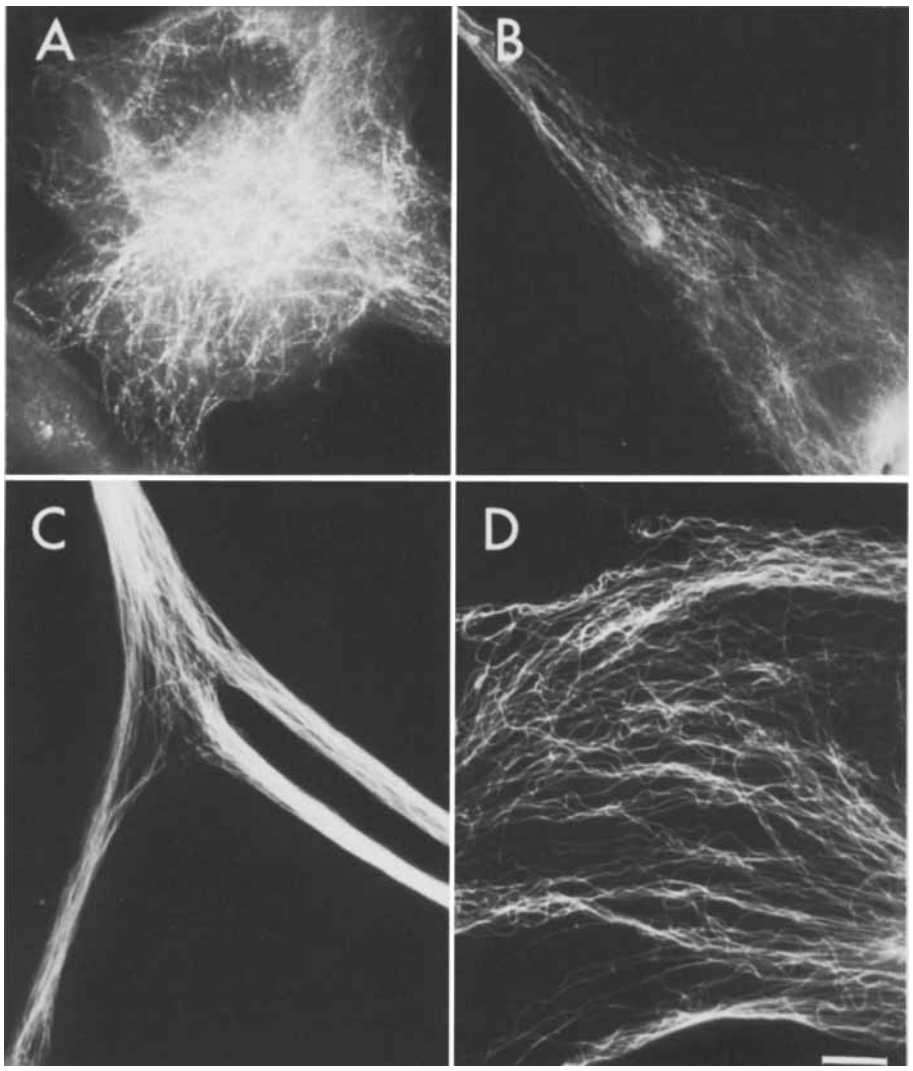

Fig. 8. *Indirect immunofluorescence staining of differentiated rat brain neuroblastoma B104 cells with anti-tubulin or anti-MAP2 antibodies.* (A) Affinity purified rabbit antibody against MAP2. (B) Affinity purified group A monoclonal antibody against MAP2. (C) and (D) Affinity purified rabbit antibodies against tubulin. Bar indicates 10 μm

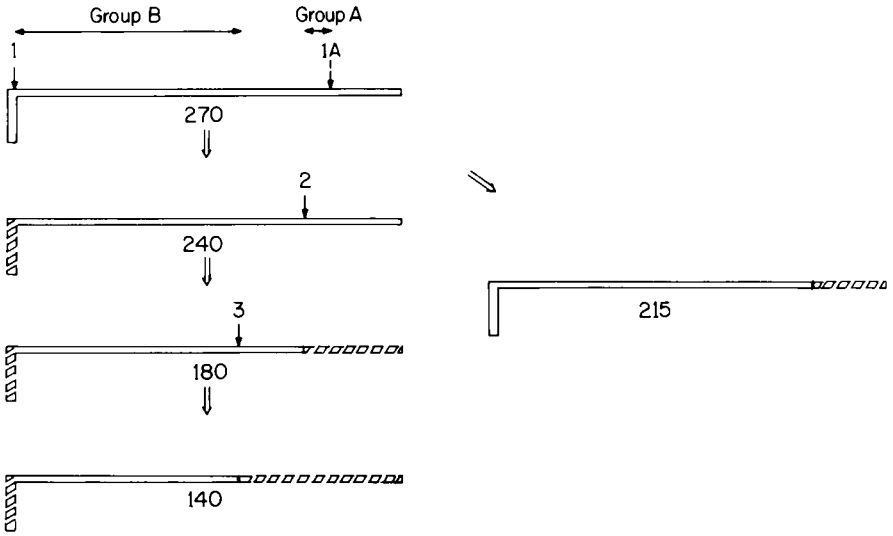

Fig. 9. *Model representing the location of the antigenic determinants along the MAP2 molecule.* In this model the microtubule-binding domain of MAP2 is illustrated by the hinge on the left side of the molecule and the projecting 'arm' region corresponds to the horizontal part of the model. The numbers above the thin arrows indicate cleavage sites of α-chymotrypsin. Numbers above the thick arrows indicate molecular mass in kDa. Unhatched area indicates the relative length of the chymotryptic fragments. The suggested location of the antigenic determinants for group A or group B anti-MAP2 monoclonal antibodies is indicated at the top of the diagram

the distal 60-kDa segment of the side arm (arrow 2, Fig. 9). This leads to the suggestion that group A antibodies react with sites located at or dependent upon the terminal 60-kDa domain(s) distal to the microtubule-binding site of MAP2. The 215-kDa fragment also reacts with group A antibodies. Peptide mapping indicates that it is not derived from the 240-kDa fragment and probably arises by an alternative cleavage pathway directly from the intact MAP2 molecule. A possible cleavage site would be distal from the microtubule-binding site (arrow 1a, Fig. 9). This would narrow down group A binding sites to an area of about 5000 Da. The second group of antibodies (group B) can still bind the smaller proteolytic products of 180 kDa and even 140 kDa which appear to be associated with the central region of MAP2 although the exact cleavage sites are still tentative.

It should be pointed out that none of our monoclonal antibodies had an effect on the binding of MAP2 to microtubules, nor did they influence microtubule assembly. Similarly, the binding to tubulin did not affect significantly the reactivity of MAP2 with the various antibodies, suggesting that microtubule-binding regions and the arm domains are functionally independent of each other. The above results are in accord with the ability of group A antibodies to visualize micro-tubules in differentiated neuroblastoma cells.

Though the number of different monoclonal antibodies tested was limited (eight clones) it is noteworthy that none of them reacted against the microtubule-binding region. If this observation reflects some of the intrinsic immunogenic reactivity of MAP2 one may infer that this area is ubiquitous and evolutionarily conserved, and thus may have a tendency to be immunologically silent. It could be suggested that the MAP2 arm region may vary in its structure in different species. On the other hand, the binding region to microtubules should display less variability since the tubulin subunits are highly conserved [37–39]. The conflicting results regarding MAP2 distribution in various sources [13,15–20] may result from differences in the specificity for the antigenic determinant(s) to which those antibodies were produced. The use of monoclonal antibodies should aid clarification of homologies of various MAP2 determinants derived from various sources and may also provide a useful tool to study MAP2 interactions with other cytoskeletal elements of the cell.

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