

MAP3: Characterization of a Novel Microtubule-associated Protein

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ABSTRACT Using monoclonal antibodies we have characterized a brain protein that copurifies with microtubules. We identify it as a microtubule-associated protein (MAP) by the following criteria: (a) it copolymerizes with tubulin through repeated cycles of microtubule assembly *in vitro*; (b) it is not associated with any brain subcellular fraction other than microtubules; (c) in double-label immunofluorescence experiments antibodies against this protein stain the same fibrous elements in cultured cells as are stained by antitubulin; and (d) this fibrous staining pattern is dispersed when cytoplasmic microtubules are disrupted by colchicine. Because it is distinct from previously described MAPs we designate this novel species MAP3. The MAP3 protein consists of a closely spaced pair of polypeptides on SDS gels, M_r 180,000, which are present in both glial (glioma C6) and neuronal (neuroblastoma B104) cell lines. In brain the MAP3 antigen is present in both neurons and glia. In nerve cells its distribution is strikingly restricted: anti-MAP3 staining is detectable only in neurofilament-rich axons. It is not, however, a component of isolated brain intermediate filaments.

In addition to the major subunit protein, tubulin, microtubules repolymerized *in vitro* contain a set of minor polypeptides known as microtubule-associated proteins (MAPs).¹ It is becoming clearer that microtubules prepared from various tissues (1, 7, 11, 14) and various cell types (8, 15, 18, 20) differ in the MAPs they contain. Several MAPs characteristic of brain have been identified, including high molecular weight forms usually referred to as MAP1 and MAP2 (3, 13, 25, 29), and a set of related species of ~60,000 mol wt collectively known as tau (30).

In addition to these, *in vitro* assembled brain microtubules contain many other minor nontubulin components. A recent study based on detergent extraction of cultured sympathetic neurons suggested the presence of >20 MAPs in these cells (7). In a monoclonal antibody library constructed against brain MAPs we have found several antibodies that react with previously uncharacterized microtubule proteins. One of these consists of an 180,000 M_r polypeptide doublet, which satisfies several criteria for identification as a MAP and which we therefore refer to as MAP3. Unlike MAPs 1 and 2, which immunohistochemical staining with antibodies shows to be predominantly neuronally localized in brain (5, 9, 10, 16, 21,

22, 31), MAP3 is present in both glial cells and neurons. Its most individual characteristic is a preferential association, within neurons, with neurofilament-rich axons and a corresponding absence from dendrites. A preliminary report of this work has been published (21).

MATERIALS AND METHODS

Preparation of Subcellular Fractions: Microtubules were prepared from the brains of albino rats (body weight 160–180g) by the method of Karr et al. (19). After three cycles of repolymerization they were stored at -70°C until required and then depolymerized and centrifuged at 100,000 g for 30 min immediately before use. In some experiments higher cycle number microtubules were used but this made no difference in the results of the experiments reported here. We made brain intermediate filaments as previously described using 1 mM EGTA in all solutions (details in reference 23). Preliminary experiments showed that 1 mM EGTA was as effective as 10 mM EGTA in inhibiting Ca^{2+} -induced proteolysis of both microtubule and intermediate filament proteins. Synaptosomes, synaptic membranes, myelin, mitochondria, and postsynaptic densities were prepared as previously described (23). Brain actomyosin was prepared as described by Beach et al. (2).

Cell Culture: Swiss 3T3, HeLa, glioma C6, neuroblastomas B104, NB2A, N18, and NIE115 were all cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After subculture the cells were seeded onto sterile coverslips at 10^5 cells/ml and grown on for 24 h. Primary astrocytes were prepared from cerebella of newborn rats. The tissue was chopped, rinsed in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.4) then dissociated in PBS containing 0.1% trypsin

¹ Abbreviations used in this paper: IF, intermediate filament; Ig, immunoglobulin; MAP, microtubule-associated protein.

(Gibco AG, Basel) for 5 min at 37°C, collected in Dulbecco's medium containing 10% fetal calf serum, and filtered through a fine nylon mesh. The cells were washed twice with medium by centrifugation at 1,200 rpm, and the final pellet was resuspended in Dulbecco's medium containing 5% fetal calf serum. The cells were plated at a density of 4×10^5 /ml in 35-mm petri dishes containing sterile glass coverslips and maintained at 37°C in 5% CO₂ for 5 d before use. The coverslips were rinsed in PBS and fixed with either acetone or methanol (HeLa cells only) at -20°C for 5 min then immediately rehydrated in PBS.

Immunofluorescent Labeling of Cultured Cells: Before immunofluorescent staining the fixed cells were treated with PBS containing 5% fetal calf serum for 10 min to block nonspecific binding. They were then incubated with monoclonal anti-MAP3 (1:100 dilution of ascites fluid in PBS) for 4 h or, more often, overnight (16 h), then washed with PBS and incubated further with rhodamine-labeled goat anti-mouse immunoglobulin (Ig; Cappel Laboratories, Cochranville, PA) at 1:50 dilution. Double immunofluorescent labeling was carried out by incubation of the coverslips first with PBS containing 1% monoclonal anti-MAP3 and 2% polyclonal (rabbit) antitubulin. The second incubation was with one of several combinations of fluorescently labeled antibodies: rhodamine-labeled goat anti-mouse Ig (Nordic, Biogenzia Lemania, Lausanne), Texas red-labeled sheep anti-mouse Ig (Amersham International, Amersham, England) or rhodamine-labeled sheep anti-mouse Ig (Cappel Laboratories) together with fluorescein labeled goat anti-rabbit Ig (Nordic, Biogenzia Lemania) or fluorescein labeled swine anti-rabbit Ig (Cappel Laboratories Inc.). These labeled antibodies were used in pairs, each at 2% concentration in PBS containing 5% fetal calf serum to suppress nonspecific attachment of the label to the sections. Each pair was checked by our incubating it with a section that had been exposed to only either anti-MAP3 or antitubulin and confirming that there was no cross-reaction of the anti-rabbit label with the monoclonal antibody or of the anti-mouse label with the rabbit antisera. The use of several combinations reflects changes in the availability and quality of reagents during the course of this study.

Antibodies and Related Procedures: BALB/c mice were immunized with the MAP fraction from third cycle microtubules, and clonal lines of antibody-secreting lymphocyte-myeloma hybrid cells were generated by use of standard techniques (see reference 16). Positive clones were identified by screening against dot-blot of MAP proteins on nitrocellulose. The MAP species with which the antibody of each positive clone reacted was then established by immunostaining nitrocellulose electroblots of SDS gels (23) in which microtubule proteins had been separated. The original anti-MAP3 secreting clone was subcloned and five subclones were tested, all of which had identical immunochemical properties and gave identical tissue and cell staining patterns. Antibodies from a single high-producing clone were used for the remainder of this study. Immunodot assay with chain-specific typing antisera identified the antibody as IgG₁. Immunochemical and immunocytochemical procedures were exactly as previously described (16, 24).

Proteolysis Experiments: Supernatant fractions from rat brains (minus the brain stem) were prepared by homogenization of the tissue in ice-cold buffer (100 mM 2-morpholinoethanesulfonic acid, pH 6.4, 0.5 mM MgCl₂, 1 mM ethylene glycol-bis(2-aminoethylether)*N,N'*-tetraacetic acid) at a ratio of 1 ml of buffer per gram of tissue and then centrifugation in a type 35 rotor (Beckman, Geneva) at 30,000 rpm for 45 min. For experiments with calcium the supernatant was made 5 mM with respect to CaCl₂ before the microtubule proteins were added. Aliquots containing 400 µg of microtubule protein in 50 µl were added to 50 µl of brain supernatant and incubated at 37°C for various times. The reaction was stopped by our making the sample 2% in SDS and 2-mercaptoethanol and immediately heating it to 90°C for 5 min.

RESULTS

Characterization of the Monoclonal Antibody and its Antigen

The distinguishing features of the antibody are the characteristic staining pattern it gives on brain sections (see below) and its selective reaction with a subclass of brain MAP proteins (Fig. 1). On nitrocellulose blots of SDS gels, the strongest reaction was with a pair of polypeptides that migrate with an *M_r* of 180,000. The antibody also reacted more weakly with doublet bands at ~170,000 and 150,000. There was no reaction with tubulin, MAP1 or MAP2, or with MAPs of the tau group. In addition to its reaction with these polypeptides on SDS gel blots, the antibody also gave a positive reaction with

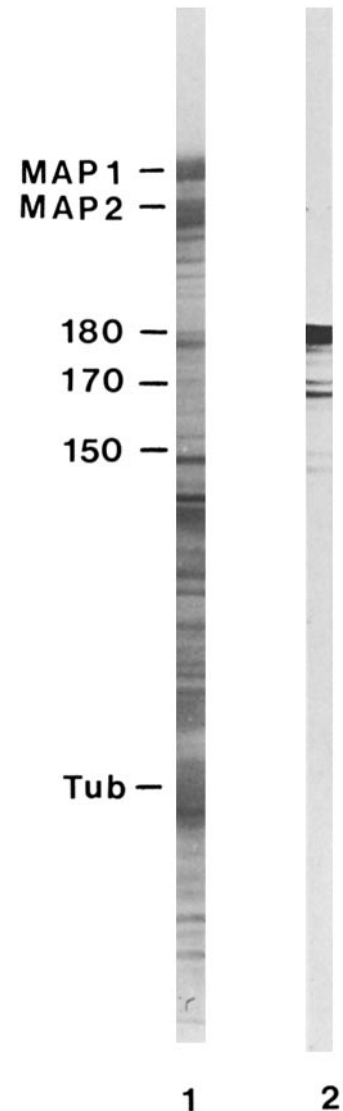


FIGURE 1 Reaction of anti-MAP3 monoclonal antibody with brain microtubular proteins. Rat brain microtubule protein was run in a broad slot on an SDS polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose sheet which was cut into several vertical strips, each with an identical content of peptide bands. Strip 1 was stained with amido black for protein whereas strip 2 was stained with anti-MAP3. The positions of MAP1, MAP2, and tubulin (*Tub*) are indicated with some molecular weights ($\times 10^{-3}$).

native MAPs (i.e., not SDS denatured) on cellulose nitrate dots.

All the bands stained by the antibody showed selective copolymerization with tubulin (Fig. 2 and Table I) and were absent from the warm supernatant to the assembled microtubules (Fig. 1, sample 5). Thus, from the brain homogenate through the first two cycles of microtubule assembly the reactive bands were progressively enriched in the microtubule fraction (compare samples 1, 4, and 8, Fig. 2) and they remained in the microtubules at the same relative level through subsequent cycles of assembly and disassembly (compare samples 8 and 11, Fig. 2). During this procedure there was a noticeable increase in the amount of material in the lower molecular weight bands relative to the 180,000 doublet (compare samples 4, 6, 9, and 12, Fig. 2*b*). This probably reflects degradation by residual proteases in the samples (see below).

Microtubular Localization

To confirm that the antigenic protein was associated with microtubules *in vivo*, we used the antibody to stain cultured cells. In one series of experiments we double labeled astrocytes in primary brain cell cultures with monoclonal anti-MAP3

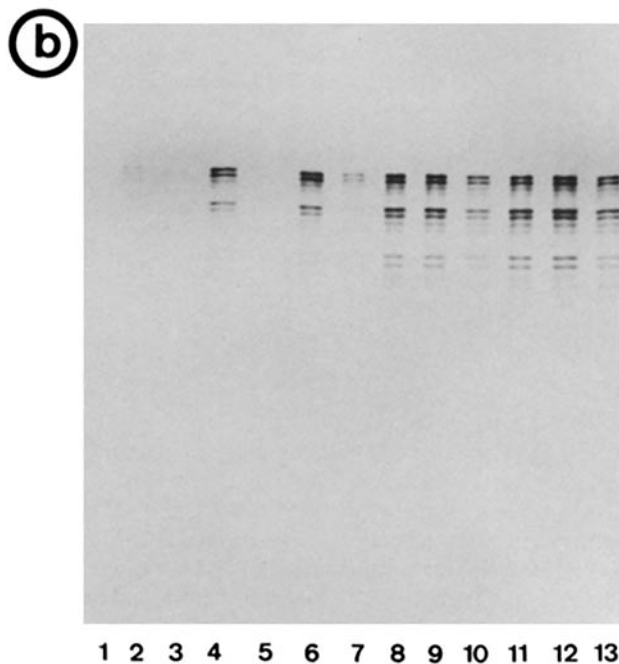
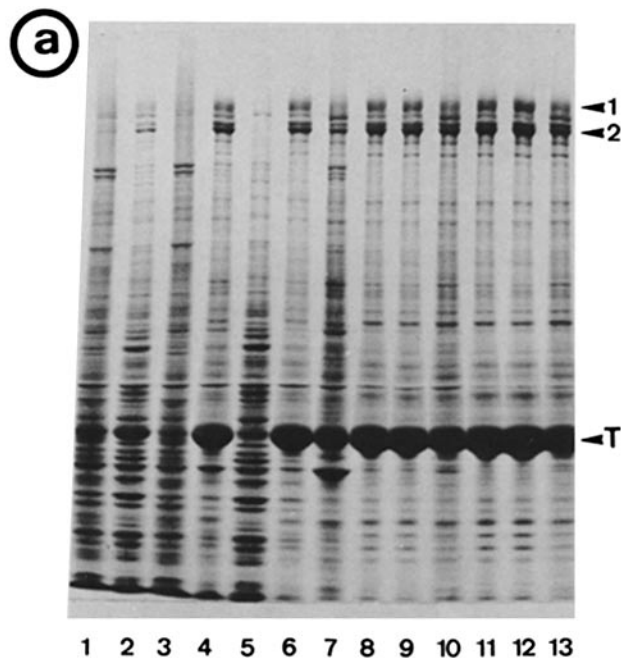


FIGURE 2 The partitioning of MAP3 during three cycles of microtubule assembly. Samples were taken at each step during the preparation of microtubules and run on SDS gels; the same amount of protein (10 μ g) was loaded in each channel. *a* shows the Coomassie Blue-stained gel on which the positions of MAP1, MAP2, and tubulin are marked (*1*, *2* and *T*). *b* shows an anti-MAP3 stained nitrocellulose blot of an identical gel. A key to the different samples appears in Table I.

and rabbit antiserum against tubulin. In all cells the distribution of MAP3 (Fig. 3, *b* and *d*) coincided with that of antitubulin stained fibers (Fig. 3, *b* and *c*). In a second series of experiments we used anti-MAP3 to stain glioma C6 cells in cultures, some of which had been exposed to colchicine before fixation. The untreated controls showed a microtubular staining pattern (Fig. 4*a*) similar to that found in the primary

TABLE I
Partitioning of MAP3 during Microtubule Assembly

Sample No.*	Sample	MAP3 status
1	Brain homogenate, 2°C	u
2	Supernatant from sample 1	w
3	Pellet from sample 1	u
4	First cycle microtubules (pellet after assembly of sample 2)	e
5	Supernatant to sample 4	u
6	Disassembled microtubules	e
7	Cold-insoluble (2°C) pellet from sample 4	d
8	Second cycle microtubules	e
9	Depolymerized microtubules from sample 8	e
10	Cold-insoluble pellet from sample 9	d
11	Third cycle microtubules	e
12	Disassembled microtubules from sample 11	e
13	Cold-insoluble pellet from sample 11	d

e, enriched; u, undetectable; d, depleted; w, weak.

* Sample numbers correspond to slot numbers in Fig. 2.

astrocytes. The colchicine treated cells were still stained by the antibody but the fibrillar staining pattern was no longer visible (Fig. 4*b*).

Origin of the Lower M_r Bands Stained by the Antibodies

In addition to the major 180,000 pair stained by the antibody, all brain microtubule preparations contained lower M_r anti-MAP3 reactive bands, the amount of which varied in different microtubule preparations. This could be explained by MAP3's being susceptible to degradation by endogenous brain proteases active during microtubule isolation. To test this, we exposed brain microtubules to separately prepared brain high speed supernatant at 37°C and looked for evidence of breakdown of the 180,000 doublet to the lower molecular weight forms. Degradation of MAP3 occurred to the greatest extent in the presence of added calcium, but also when excess EGTA was added to sequester calcium (Fig. 5*a*).

In all of the lower M_r products the doublet appearance of the immunoreactive material was preserved, which suggests that the removal of identical segments from each of the two native 180,000 M_r polypeptides at each step of the degradative process occurred. The presence of these anti-MAP3 reactive fragments in freshly prepared brain microtubules was not eliminated by EGTA or by other inhibitors of the major categories of proteases, including phenylmethylsulfonyl fluoride, *p*-hydroxymercuribenzoate, *o*-phenanthroline, and leupeptin.

Cellular Distribution of MAP3

We examined a variety of cell lines for evidence of MAP3 content. In one series of experiments brain microtubules and brain homogenate (serving as positive controls) were run alongside homogenates of the various test cells in a single slab SDS-gel. The entire gel was blotted onto cellulose nitrate and stained with the antibody. The results of one such experiment are shown in Fig. 6. In this case two identical gels were run and blotted, one of which was stained with Coomassie Blue to reveal the pattern of major proteins in the different cell types (Fig. 6*a*) and the other blotted onto nitrocellulose and stained with anti-MAP3 (Fig. 6*b*). With respect to protein

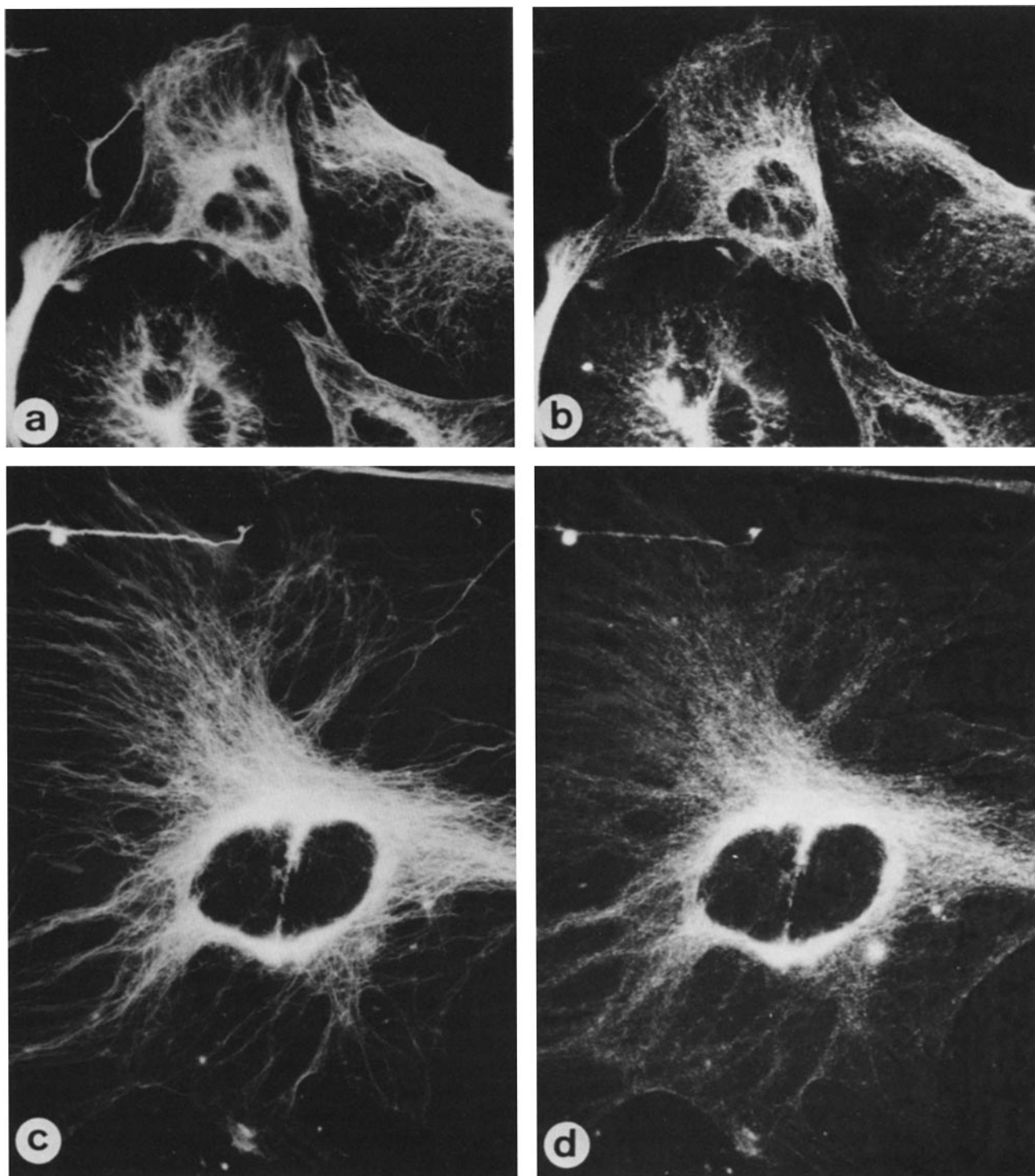


FIGURE 3 Immunofluorescent double labeling of cells in primary brain cell cultures by polyclonal antiserum against tubulin (a and c) and monoclonal antibodies against MAP3 (b and d).

content the various neuroblastomas (slots 7 to 16) are quite similar to one another as well as to the glioma cells (slots 5 and 6) and to brain homogenate (slots 3 and 4). However, in MAP3 content there are marked differences between these various cell lines (Fig. 6*b*). When brain homogenate (slots 3 and 4) are used as a standard, it is clear that glioma C6 cells are relatively rich in MAP3 (slots 5 and 6). This is also evident by immunofluorescent staining (Fig. 4*a*). Among the neuroblastoma cell lines there is a marked contrast between B104,

which does produce MAP3, and all of the other neuroblastomas tested (NB2A, N-18, and NIE-115), in which the antigen is undetectable. In B104 cells the level of MAP3 immunoreaction relative to total cellular protein is markedly increased when the cells are induced to differentiate by application of cyclic AMP (compare slots 7 and 8 and 9 and 10, Fig. 6*b*).

The experiment shown in Fig. 6 is one of several in which we compared microtubules and brain homogenate by use of anti-MAP3. A consistent feature of these experiments is the

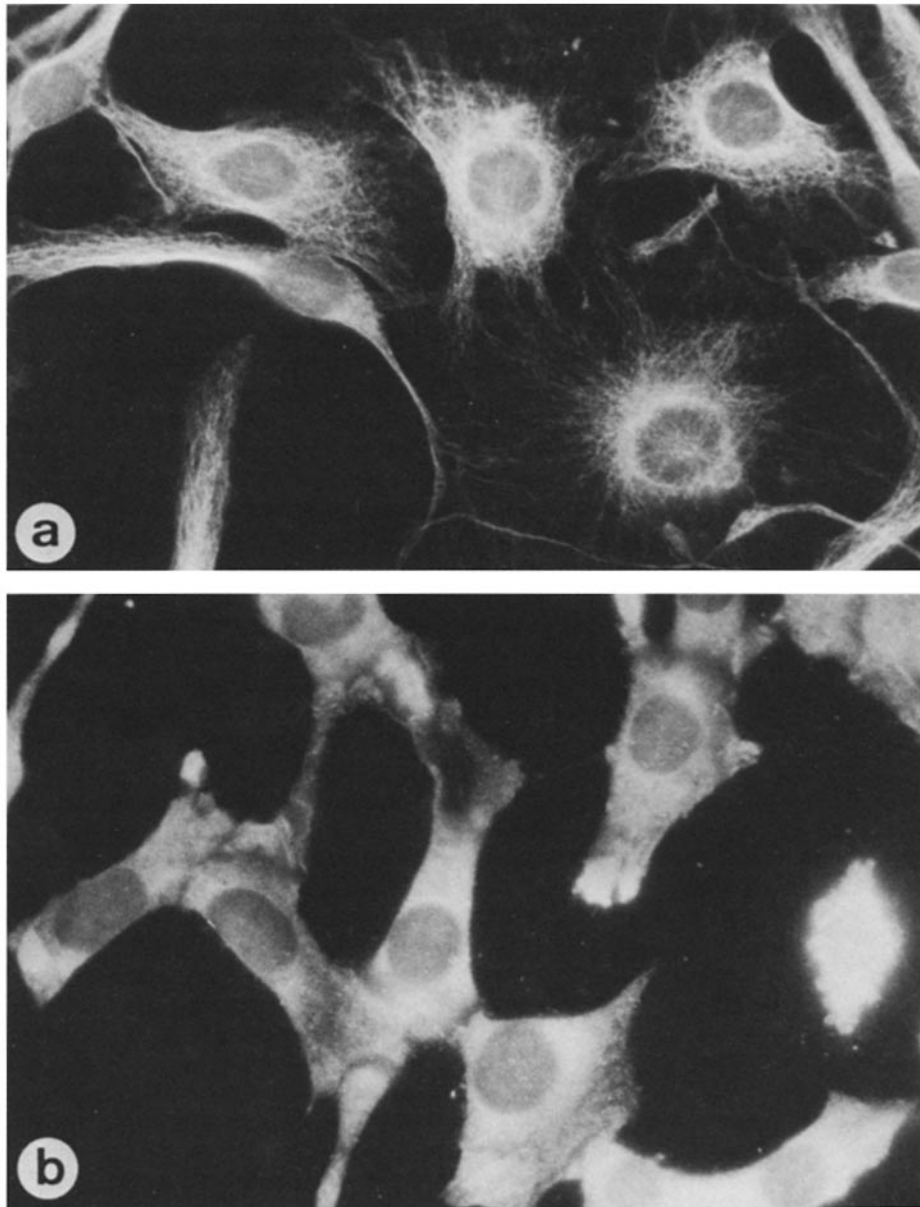


FIGURE 4 Glioma C6 cells stained with monoclonal anti-MAP3. The cells in *a* were in an untreated control culture whereas the cells in *b* were in a culture that was treated with colchicine (1×10^{-6} M) for 2 h before fixation.

evident specificity of the monoclonal antibodies for MAP3. Thus, in brain homogenate no proteins other than the 180,000 doublet react with anti-MAP3 (Fig. 6*b*, slots 3 and 4). We also observed by comparing anti-MAP3 reactive material in cell lines with that in repolymerized brain microtubules, the relative lack of lower M_r degradation products. For example, the very strong 180,000 MAP3 doublet in C6 cells is accompanied by a much weaker immunoreactive doublet at 170,000 and no further breakdown products (Fig. 6*b*, slot 5). In comparison, brain microtubules in the same gel give less staining of the 180,000 doublet but much more intense lower M_r bands (slot 1).

In addition to neuroblastoma and glioma we also examined two non-neural cell lines, Swiss 3T3 and Hela cells. Neither of these contained immunoreactive MAP3 when cell homogenates were tested by solid-phase immunoassay with anti-MAP3. Each of these cell lines was also checked for immu-

nofluorescent staining by anti-MAP3, but there was no detectable reaction. We also tested a series of other brain subcellular fractions by staining SDS-gel blots with anti-MAP3. In none of these, actomyosin, myelin, mitochondria, synaptic membranes, and postsynaptic densities, was MAP3 detectable.

Distribution of MAP3 in Brain

In brain sections MAP3 shows a distinctive distribution, being restricted to astroglial cells and neurofilament-rich axons. This is well demonstrated in two tissues, the cerebellum and the hippocampus.

In the cerebellar cortex (Fig. 7) both types of staining are already apparent at low magnification (Fig. 7, *a* and *b*). Myelinated axons in the white matter are strongly labeled and the radially oriented fibers of the Bergmann glial cells in the

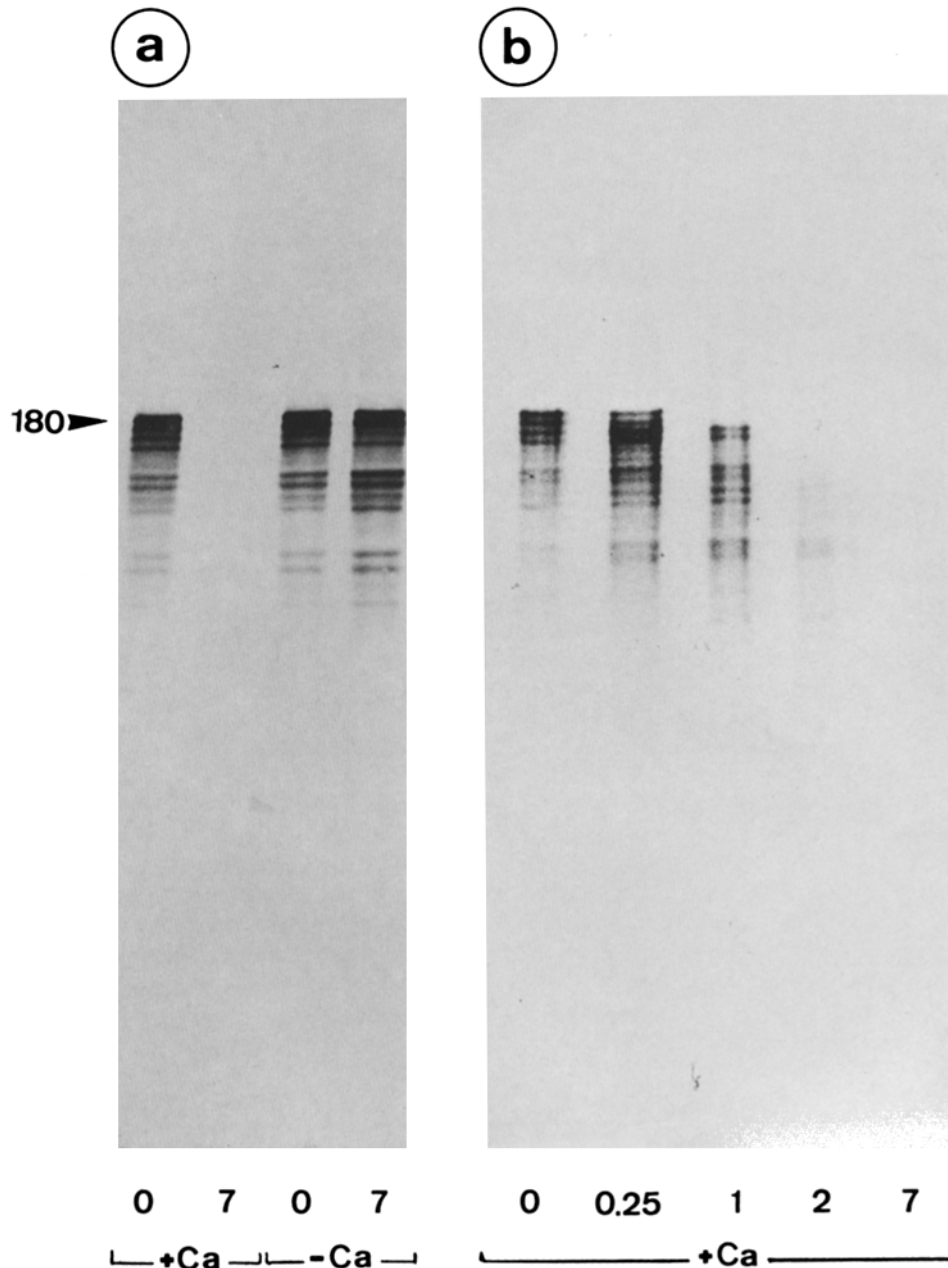


FIGURE 5 Anti-MAP3-stained SDS gel blots of total proteins from microtubules that had been incubated with brain supernatant for the times indicated (in hours) beneath each channel. *a* shows the different effects of incubation with or without Ca^{2+} for 7 h. *b* shows the time course for degradation in the presence of Ca^{2+} . 180, 180,000 mol wt.

outer molecular layer are also prominently stained. Closer examination of the white matter in transverse (Fig. 7*c*) or longitudinal (Fig. 7*d*) section confirms that the staining is present in the axoplasmic cores of all the white matter axons. The myelin sheaths around the axons (arrowheads) are unstained.

At higher magnification the stained Bergmann glial fibers are clearly resolved (Fig. 7*e*), and the lack of staining of either axons or dendrites in the neuropil above the Purkinje cells is also apparent. Outside of the white matter only one set of neuronal processes shows any MAP3 staining. These are the terminal arborizations of basket cell axons which, as shown in Fig. 7*f*, are labeled by anti-MAP3 as they make their typical basket-like enclosure around the cell bodies and initial axons segments of Purkinje cells.

In the hippocampus, the anti-MAP3 staining pattern is also distinctive. Throughout, there is staining of scattered small cell (Fig. 8*a*), which at higher magnification are revealed as astroglia (Fig. 8, *b* and *c*). Neuronal MAP3 immunoreactivity in the hippocampal formation is limited to a single tract, the mossy fibers. This tract consists of the axons of granule neurons in the dentate gyrus which terminate in synapses onto proximal regions of the apical dendrites of pyramidal neurons in the CA3 area of the hippocampal gyrus. The anti-MAP3 staining in the hippocampus followed exactly this pattern, showing strong staining of axons arising in the dentate gyrus (Fig. 8*b*) and running as a tract just superior to the CA3 pyramidal cell bodies. The staining became progressively weaker along the course of the CA3 region (Fig. 8*a*), reflecting the progressive thinning out of the tract as more axons ter-

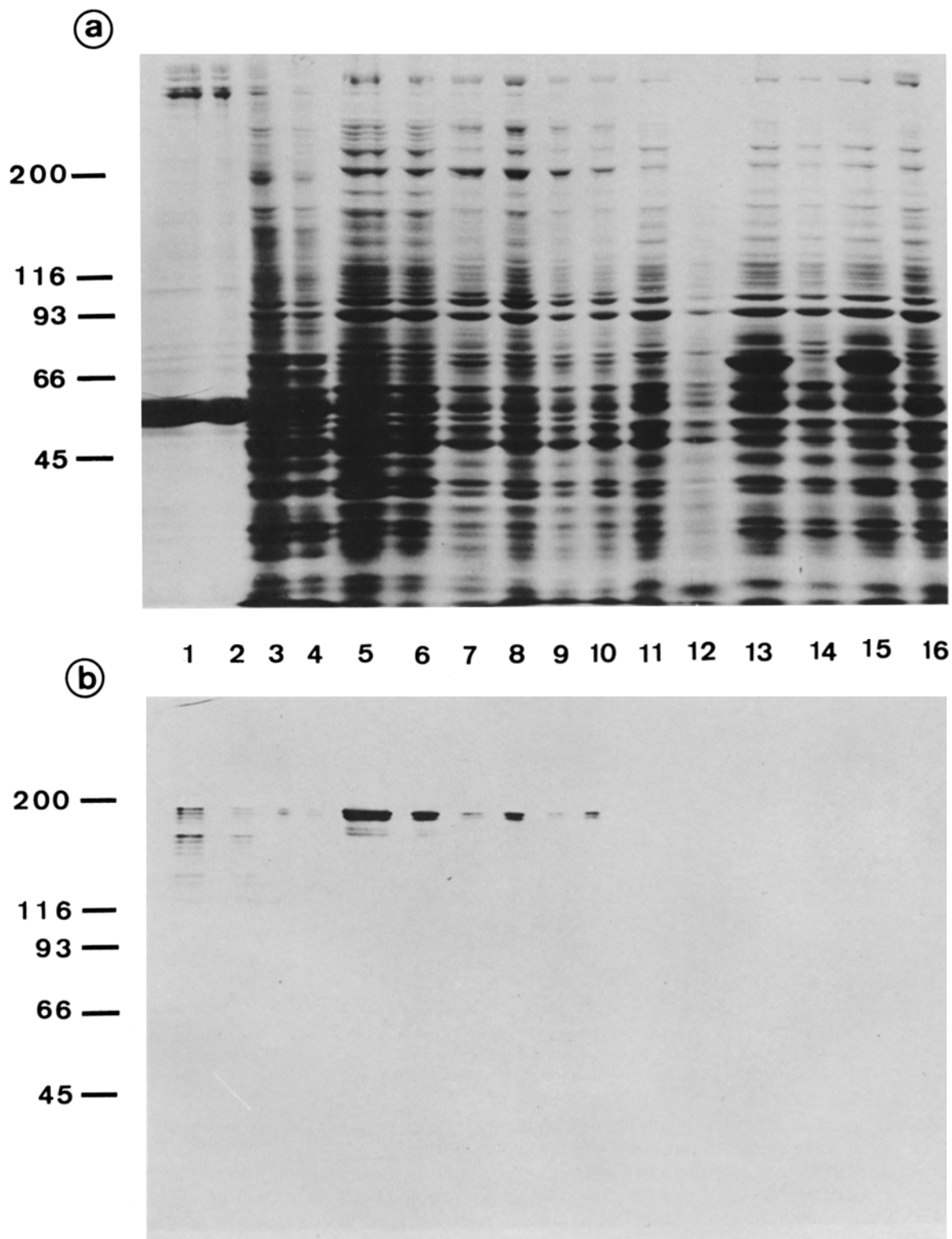


FIGURE 6 Tests for the presence of MAP3 in neural cell lines. *a* shows a gel stained with Coomassie Blue for proteins; *b* shows an anti-MAP3-stained nitrocellulose blot from a gel run in parallel. Channels 1, 3, 5, 7, 8, 11, and 13–16 were loaded with 40 μ g of protein, the others with 20 μ g. The different channels contain proteins of (1 and 2) brain microtubules, (3 and 4) brain homogenate, (5 and 6) glioma C6 (7 and 9) neuroblastoma B104 grown without cyclic AMP, (8 and 10) B104 grown with cyclic AMP (11 and 12) neuroblastoma N2a (13 and 14) neuroblastoma N118, and (15 and 16) neuroblastoma NIE115.

minate. Within the tract itself both the stained fibers and glial cell bodies are visible (Fig. 8*d*).

The pattern of nerve cell staining in brain sections by anti-

MAP3 parallels the distribution of intermediate filaments (IFs). Thus myelinated axons, cerebellar basket cell axons, and hippocampal mossy fiber axons are all rich in neurofila-

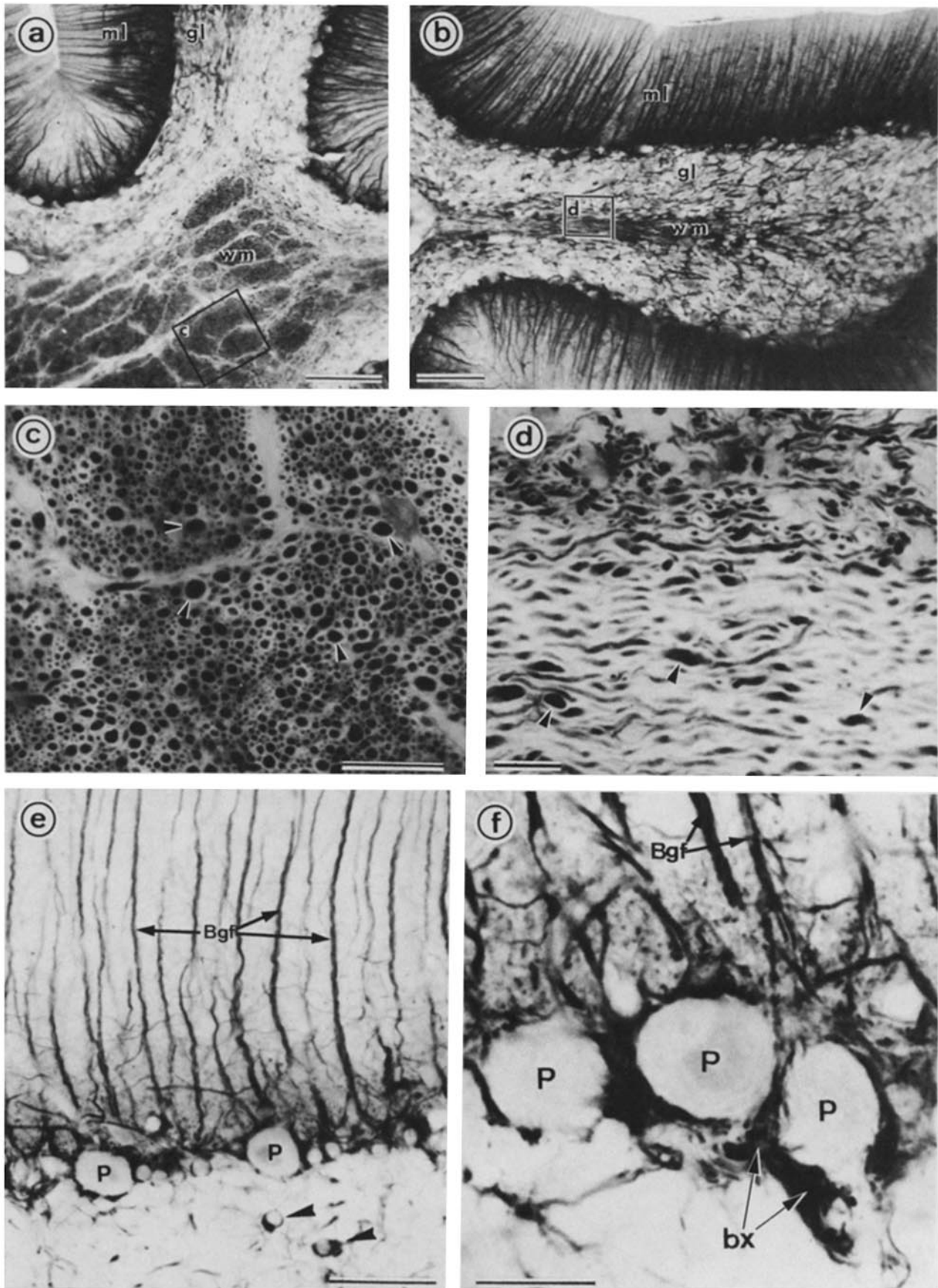


FIGURE 7 Sections of cerebellar cortex stained with anti-MAP3. (a) Central area showing white matter (*wm*). The granule cell layer (*gl*) and the molecular layer (*ml*) are identified. (b) A single folium. (c) The area labeled *c* in *a*. (d) The area labeled *d* in *b*. (e) The Purkinje cell layer showing stained Bergmann glial fibers (*Bgf*) and stained astrocytes among the unstained granule cell bodies (arrowheads). (f) Close-up of Purkinje cell bodies (*P*) to show the stained basket cell axon terminal arborizations (*bx*) around Purkinje cell initial axon segments. Bars, (a and b) 100 μm ; (c and d) 25 μm ; (e) 50 μm ; and (f) 20 μm .

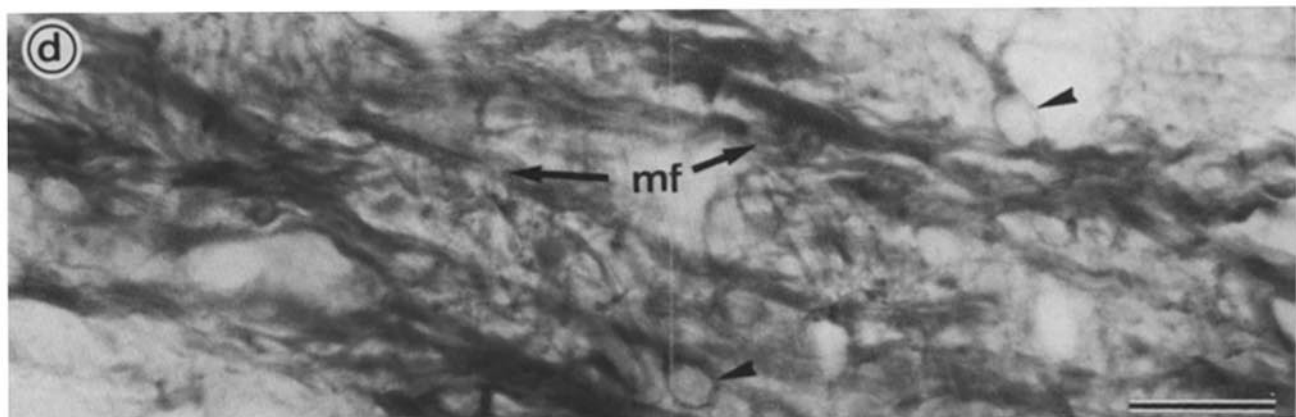
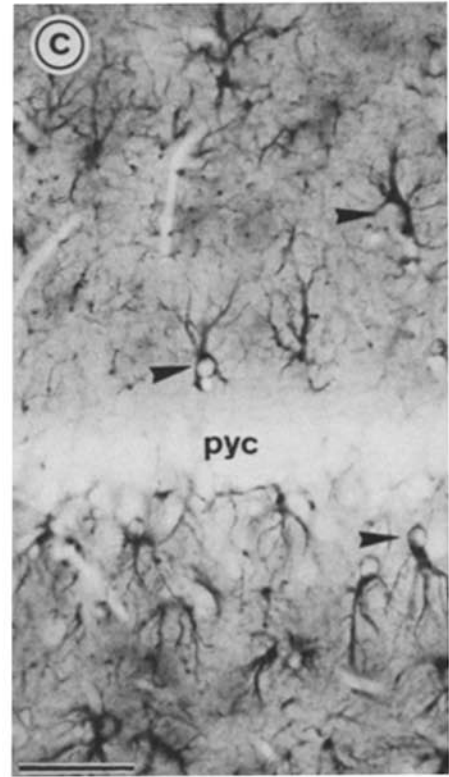
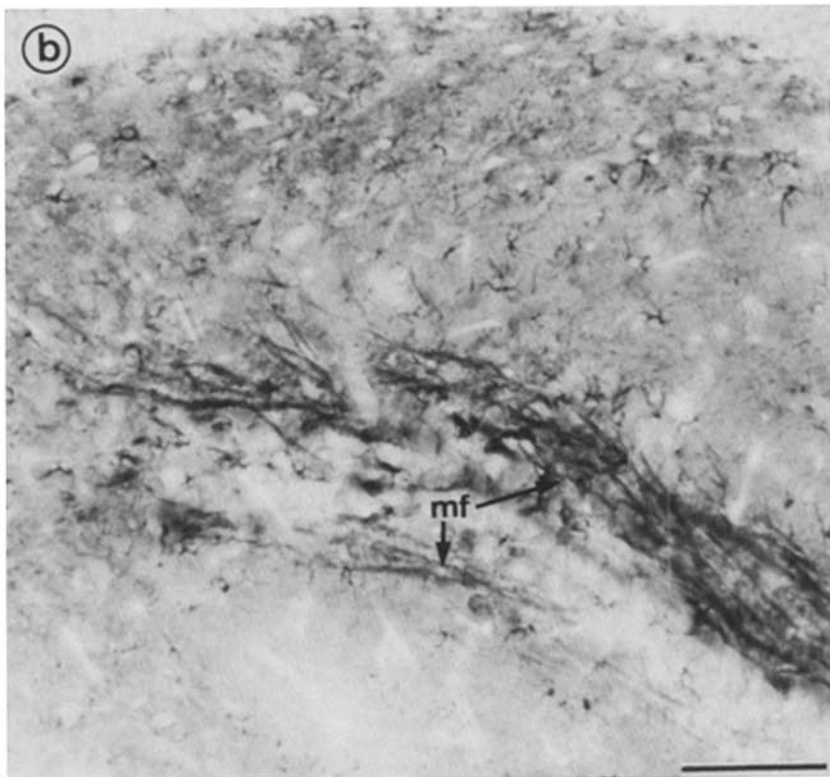
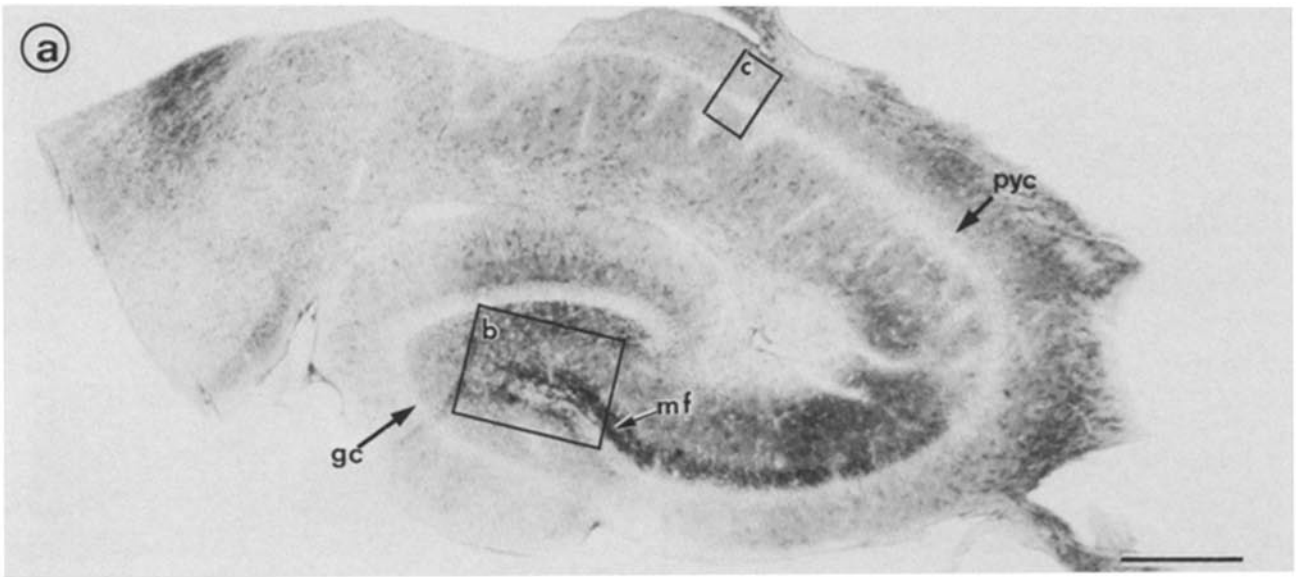


FIGURE 8 Hippocampus stained with anti-MAP3. (a) An entire section with the mossy fiber tract (*mf*) and the layer of granule cells (*gc*), from which these axons arise labeled. The areas covered by *b* and *c* are also indicated. (b) The mossy fiber tract at its area of origin in the dentate gyrus. (c) Part of the CA1 portion of the hippocampal gyrus around the pyramidal cell layer (the layer of pyramidal cell bodies is labeled '*pyc*'). Some of the stained astrocytes are indicated with arrowheads. (d) Stained fibers (*mf*) in the mossy fiber tract. Stained glial cells are indicated (arrowheads). Bars, (a) 0.5 mm; (b) 100 μ m; (c) 50 μ m; and (d) 25 μ m.

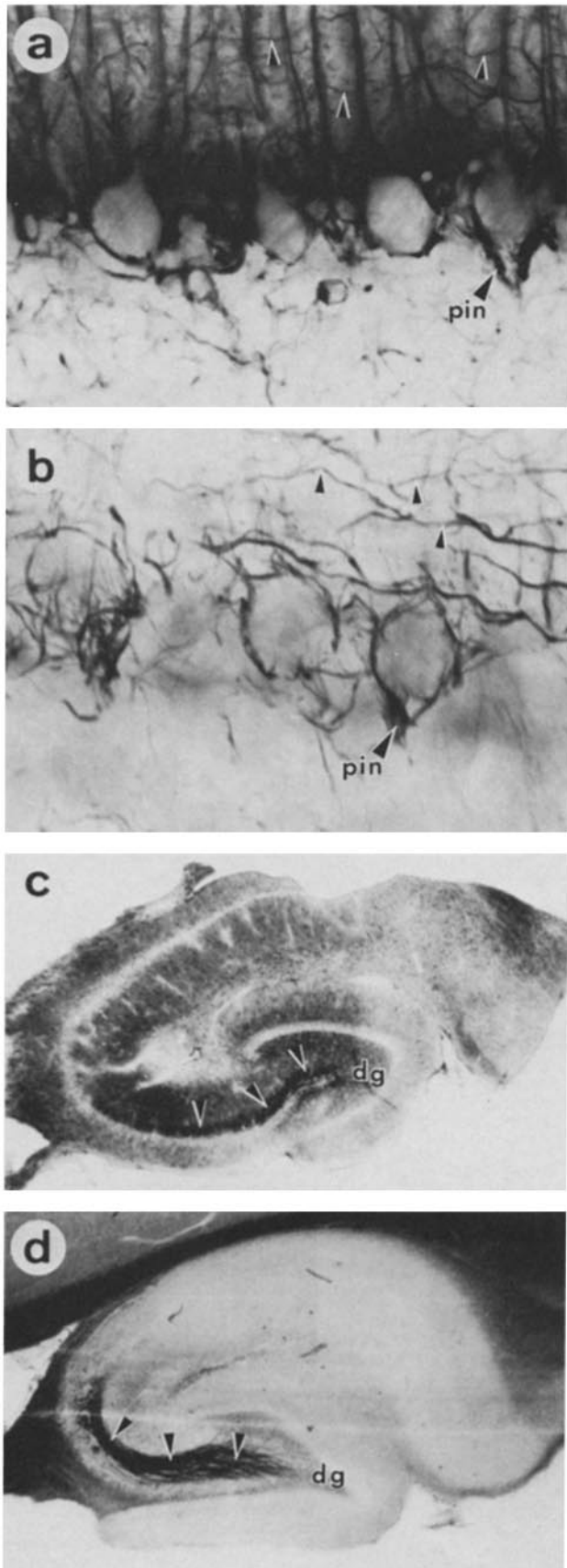


FIGURE 9 Comparison of anti-MAP3 and anti-neurofilament (anti-NF) staining patterns. (a and b) Purkinje cell layer of the cerebellar cortex stained with anti-MAP3 (a) or anti-NF (b). Stained basket axon terminals forming typical pinneau (*pin*) formations around

ments (Fig. 9 and reference 32), and the astroglial cells stained by anti-MAP3 are rich in glial filaments (6, 23, 27). We therefore considered the possibility that, in addition to its relationship with microtubules, MAP3 might also be associated with brain intermediate filaments. To test this, we made brain IF preparations that contained both neuronal and glial filaments (12, 23) and examined them to see whether they contained MAP3. Fig. 10 shows two SDS-gel blots of brain IFs (slots 1 and 2) and two blots of brain microtubules to serve as controls (slots 3 and 4). The microtubule blot shows the usual pattern of MAP3-reactive bands. By contrast the IF blot, although loaded with the same amount of protein, contains no anti-MAP3 reactive material (slot 1). The brain IF preparations used in this study were made in the presence of 1 mM EGTA and protease inhibitors (for details see reference 23) so the absence of MAP3 from these preparations cannot be attributed to proteolytic degradation.

DISCUSSION

The monoclonal antibody described in this study reacts with a protein antigen, which satisfies several criteria for its identification as a MAP. First, MAP3 is present in brain microtubules but absent from all other subcellular fractions. Second, it repeatedly copolymerizes with tubulin through multiple cycles of microtubule assembly and disassembly. Third, in cultured cells anti-MAP3 decorates the same set of fibrous elements that are stained by antitubulin. Fourth, in cells whose microtubules have been disrupted with colchicine anti-MAP3 staining loses its fibrillar pattern.

The MAP with which our antibodies react is novel in that it does not correspond to the previously characterized brain microtubular proteins MAP1, MAP2, or tau. It is also distinct from the 210,000 mol wt Hela cell MAP (8), since MAP3 immunoreactivity is absent from Hela cells by both immunohistochemical and immunofluorescence tests. MAP3 is also distinct from another recently described MAP of ~200,000 *M_r* (18) because the latter is limited to microtubules of the mitotic spindle and is apparently absent from interphase microtubules. On the contrary, anti-MAP3 stains interphase microtubules in cultured cells.

Most of the MAP3 protein in brain consists of a pair of polypeptides close in size. Like other high molecular weight MAPs (16, 26, 28), both components of the MAP3 doublet are highly susceptible to proteases present in brain supernatant, particularly in the presence of calcium. EGTA markedly slowed the degradation of MAP3 180,000 by brain supernatant but it did not totally eliminate the breakdown products from brain microtubules, and neither did any of a variety of protease inhibitors. Thus, it seems possible that these breakdown products may already be present in brain, produced through the *in vivo* activity of the same calcium-stimulated protease that can be demonstrated in brain supernatant *in vitro*. Such a scheme has already been suggested for MAPs 1 and 2 on the basis of similar data (26).

The MAP3 antigen occurs in both neurons and glia, which

Purkinje cell initial axons segments are indicated. The stained preterminal axons of basket cells are identified by arrowheads. (c and d) Hippocampus slices stained with anti-MAP3 (c) or anti-NF (d). The mossy fiber tracts arising from the dentate gyri (*dg*) are marked with arrowheads. The background coloration with anti-MAP3 is the result of astrocyte staining.

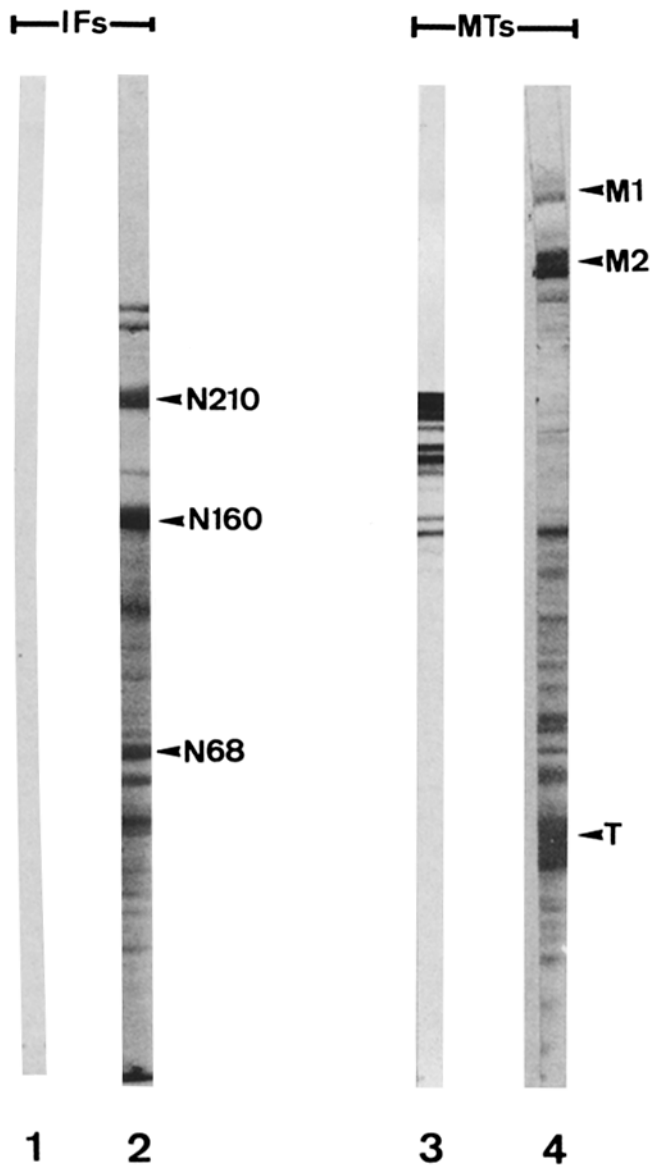


FIGURE 10 Comparison of the MAP3 contents of brain IFs and microtubules (MTs). Strips 1 and 2 were cut from a single nitrocellulose blot of an SDS gel in which brain IFs were run. Similarly, strips 3 and 4 were from a single blot of a brain MT gel. Strips 2 and 4 were stained for protein with amido black. Strips 1 and 3 were stained with anti-MAP3 under identical conditions. Gel loadings were 10 $\mu\text{g}/\text{mm}$ in 0.8-mm thick slots.

raises the possibility that one component of the 180,000 doublet might be present in neurons and the other in glia. However, this is unlikely to be the case because both components are present in glioma (C6) as well as neuroblastoma (B104) cells.

In neurons MAP3 has a highly restricted cytoplasmic distribution. Unlike MAP1, which is more concentrated in dendrites than axons (16, 17, 31), and MAP2, which appears to be exclusively located in the dendrites of brain neurons (4, 5, 9, 10, 19, 31), MAP3 appears to be completely absent from dendrites and is instead exclusively located in neurofilament rich axons. In this respect our monoclonal antibodies against MAP1, MAP2, and MAP3 act as controls for one another, each being IgG₁ immunoglobulins (16) and each giving its own distinctive staining pattern. The very different intraneu-

ronal staining by these three monoclonal antibodies suggests that each of the MAPs they recognize is associated with a different population of microtubules in vivo. For example, in the cerebellar cortex, strong MAP3 immunoreactivity is present in all the myelinated axons of the white matter. Many of these MAP3-positive axons belong to Purkinje cells, in which we have recently demonstrated that MAPs 1 and 2 are differently distributed (16). It thus appears that there are at least three different types of microtubules in the principal neurons of the cerebellar cortex, each characterized by different MAP species.

MAP3 is codistributed with neurofilaments in nerve cells; it is present only in neurofilament rich axons, and within those axons immunoreactive MAP3 first appears just beyond the initial axon segment in just the same way as neurofilament antigen (Fig. 9). This suggests that neuronal MAP3 is intimately connected with neurofilaments, despite its being an integral component of isolated microtubules but not of isolated neurofilaments. In the developing brain, however, MAP3 is transiently expressed in growing axons from which it is absent in the adult. At least some categories of such axons do not contain neurofilaments either during development or in the adult brain. For example the axons of cerebellar granule cells are rich in MAP3 between the first and third postnatal weeks but thereafter MAP3 is not detectable in these cells (4).

We thank Dr. K. Fujiwara for a gift of antitubulin, Dr. D. Schubert for providing B104, and Dr. D. Monard for providing mouse neuroblastoma cell lines. Gundula Pehling provided technical assistance and the photographic work was performed by M. Ackermann and E. Peter.

Received for publication 12 March 1984, and in revised form 12 October 1984.

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