

# Microtubule-associated protein 1B: Identification of a major component of the neuronal cytoskeleton

(monoclonal antibodies/immunofluorescence microscopy/peptide mapping/rapid axonal transport)

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**ABSTRACT** The major nontubulin proteins in purified brain microtubules are high molecular weight species traditionally classified into two groups known as microtubule-associated proteins 1 and 2 (MAP 1 and MAP 2). In an earlier study, we found that MAP 1 consisted of a complex of polypeptides and we characterized the highest molecular weight species—MAP 1A—with the use of a monoclonal antibody. In the current report, we describe four monoclonal antibodies raised against electrophoretically purified MAP 1B. All of the antibodies reacted exclusively with this protein. Together with peptide mapping, these results indicated that MAP 1B was structurally distinct from the other MAPs. Another distinctive property of MAP 1B was that most of it remained soluble during microtubule polymerization, resulting in an extreme underestimate of its abundance in the brain. Immunofluorescence microscopy of rat brain sections and cultured rat brain cells indicated that compared to MAP 1A and MAP 2, MAP 1B was particularly prominent in axonal as well as dendritic processes. Together, these data indicate that MAP 1B is a major, previously undescribed component of the neuronal cytoskeleton.

Cytoplasmic microtubules are composed of two classes of protein, the tubulins, which are the principal structural subunits, and the microtubule-associated proteins, or MAPs. The major MAPs in brain are high molecular weight (HMW) proteins that appear as arms that protrude from the outer walls of microtubules (1–3). These proteins have been separated into two biochemically distinct fractions known as MAP 1 and MAP 2 (3, 4).

MAP 1 and MAP 2 can be further resolved into multiple electrophoretic species that we have referred to as MAP 1A, 1B, 1C, 2A, and 2B (5). MAP 2A and MAP 2B are immunologically cross-reactive and have, so far, proven to be almost indistinguishable in biochemical properties (for review, see ref. 6). We recently described a monoclonal antibody raised against MAP 1A that reacted exclusively with this protein (5), suggesting that the MAP 1 polypeptides might not be as closely related as the MAP 2 species. However, the possibility that MAP 1B and MAP 1C represented fragments or otherwise modified forms of MAP 1A could not be ruled out.

MAP 1A and the MAP 2 polypeptides occupy distinct subcellular compartments and are differentially distributed among different cell types. MAP 2 was primarily concentrated in the dendritic processes of neuronal cells (5, 7–10). MAP 1 was considerably more widespread than MAP 2. MAP 1A was present in axonal as well as dendritic processes, glial cells, and a wide range of nonneuronal cells (5, 11). MAP 1A and MAP 2 were distinctly distributed within even the same cell (5, 12, 13), suggesting that the proteins serve distinct functions.

The present study represents a continuation of our effort to characterize the microtubule-associated cytoskeleton of neuronal cells. Our aim was to determine the structural relationship of MAP 1B to the previously described MAPs. We report that MAP 1B is distinct from all of the other MAPs, as judged by biochemical and immunological criteria, and, indeed, represents a unique and abundant component of both the dendritic and the axonal cytoskeleton.

## MATERIALS AND METHODS

**Microtubule Purification.** Microtubules were isolated with the aid of taxol from calf brain gray and white matter and from whole 7-day-old rat brain, as described (7).

**Electrophoretic Techniques.** NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis was performed according to the method of Laemmli (14). A modification of this method was also used in which NaDodSO<sub>4</sub> was omitted from both the stacking and separating gels, and 2 M urea was added to the separating gel. Gels were stained with Coomassie brilliant blue R250 (see ref. 3) or ammoniacal silver (15). For peptide mapping, microtubule proteins purified from white matter were resolved by electrophoresis on 0.75-mm thick, 4% NaDodSO<sub>4</sub>/urea gels. The entire HMW MAP region was excised, placed lengthwise along the top of a 15% polyacrylamide gel, and subjected to peptide mapping using chymotrypsin (Worthington) or *Staphylococcus aureus* V8 protease (Miles) (16).

**Anti-MAP Antibodies.** Polyclonal anti-MAP 2 and monoclonal anti-MAP 1A have been described (5, 8, 9, 11, 12, 17). Production of monoclonal antibodies to MAP 1B was performed following the procedure used for MAP 1A (5). The antigen was MAP 1B purified from bovine white matter microtubules (7) by electrophoresis on 4% NaDodSO<sub>4</sub>/urea gels.

Antibodies from four independent clones are described here. We refer to the antibodies as MAP 1B-1, 1B-2, 1B-4, and 1B-5. The antibodies were all found to be IgG1s (5) and were distinguishable by their electrophoretic mobility as well as other immunochemical and immunocytochemical properties. A fifth antibody with unique properties (MAP 1B-3) will be described elsewhere.

**Immunological Techniques.** Conditioned hybridoma culture medium and antiserum to MAP 2 diluted 1:100 in phosphate-buffered saline were used as primary antibodies. Secondary antibodies were all sheep IgG fractions obtained from Cappel Laboratories, Cochranville, PA. Immunoblotting and immunofluorescence microscopy of brain sections

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Abbreviations: HMW, high molecular weight; MAP, microtubule-associated protein.

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and of primary cultures of whole newborn rat brain cells were performed as described (5, 12).

## RESULTS

**Structural Comparison of MAPs.** We originally identified MAP 1B as an electrophoretic species in microtubules isolated from bovine white matter (5). Fig. 1 shows an electrophoretic gel of white matter and gray matter microtubules prepared under identical conditions to compare the MAP 1 polypeptide composition directly. MAP 1B and MAP 1C were considerably more abundant in the white matter than in the gray matter microtubules, though both bands could be detected at low level in the gray matter preparation as well.

To learn more about the relationship of MAP 1B to the other MAPs, we prepared a series of monoclonal antibodies to MAP 1B. Fig. 2 shows immunoblot analysis of white matter microtubules performed with four different antibodies. All of the antibodies were specific for MAP 1B when tested against purified microtubules (Fig. 2) or unfractionated brain proteins (see Fig. 4).

The absence of cross-reactivity with the other HMW MAPs suggested that MAP 1B contained regions of structural dissimilarity from these proteins. To investigate this possibility further, peptide mapping was performed. Digestion was conducted with chymotrypsin and V8 protease over a range of concentrations (0.25–2.5  $\mu\text{g}$  per lane). A representative result is shown in Fig. 3. The fragment patterns obtained for MAP 1A and MAP 1B were almost totally distinct. In addition, the sensitivity to proteolysis was very different, as

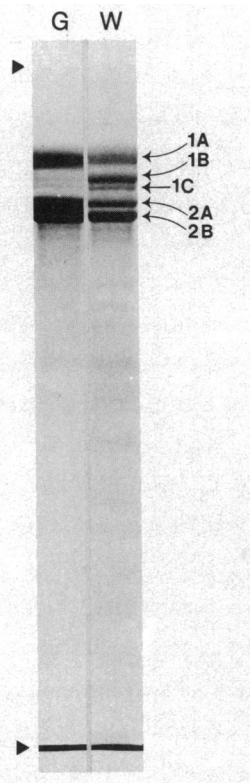


FIG. 1. Comparison of HMW MAPs purified from gray matter and white matter. NaDodSO<sub>4</sub>/urea gel (4% polyacrylamide) stained with Coomassie brilliant blue, showing purified microtubules from calf cerebral cortex gray matter (G) and corpus callosum white matter (W), prepared identically (7) and loaded at the same volume. A small amount of gray matter was intentionally included with the white matter preparation to show MAP 2 bands. Arrowheads indicate top and dye front of gel. Tubulin is found in dye front.

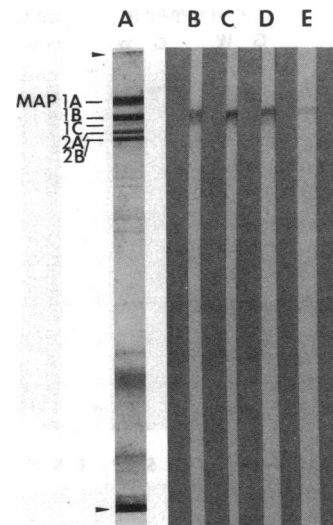


FIG. 2. Monoclonal antibodies to MAP 1B. White matter microtubule proteins were separated on a NaDodSO<sub>4</sub>/urea gel (4% polyacrylamide) as in Fig. 1 and either stained with Coomassie brilliant blue (lane A) or electrotransfered to nitrocellulose (lanes B–E). The paper was cut into strips, which were stained with anti-MAP 1B antibodies: “MAP 1B-1” (lane B), “MAP 1B-2” (lane C), “MAP 1B-4” (lane D), “MAP 1B-5” (lane E). Arrowheads denote top and dye front of gel.

judged by the extent of fragmentation as a function of protease concentration (not shown). In contrast, the fragment patterns for MAP 2A and MAP 2B were extremely alike (see ref. 19) and distinct from those of MAP 1A and MAP 1B. Analogous results were obtained with chymotrypsin (not shown).

**Relative Abundance of MAPs in Brain Tissue and Purified Microtubules.** Analysis of purified microtubules appeared to indicate that MAP 1B was primarily localized in white matter (Fig. 1). To characterize the distribution of the protein

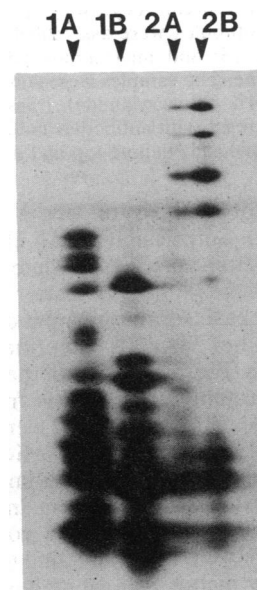


FIG. 3. Peptide mapping of MAPs. Purified white matter microtubules were subjected to NaDodSO<sub>4</sub>/urea electrophoresis as in Figs. 1 and 2. The entire HMW MAP region of the gel was excised, and the proteins were subjected to proteolysis with V8 protease in a second gel (16), shown here stained with silver stain. Fragments of MAP 1C were undetectable due to resistance of this protein to proteolysis (5) and low abundance (Figs. 1 and 2).

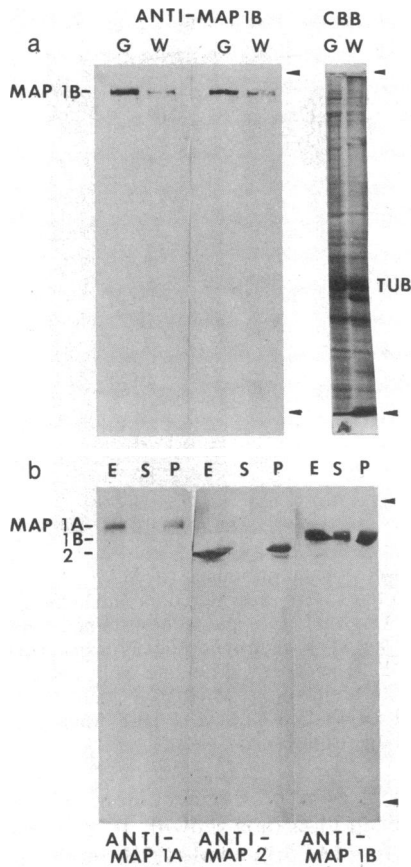


FIG. 4. Immunoblot analysis of MAPs in brain tissue and of stages in microtubule isolation. (a) Calf brain cerebral cortex (G) and corpus callosum (W) were dissolved in NaDodSO<sub>4</sub> sample buffer and subjected to NaDodSO<sub>4</sub> gel electrophoresis (7% polyacrylamide). Each lane represents 2 mg of brain tissue, wet weight. The blot on the left was performed with antibody MAP 1B-2 and the blot on the right was performed with MAP 1B-4. CBB, same samples stained with Coomassie brilliant blue; TUB, tubulin. (b) Stages in microtubule purification from rat brain. A cytosolic extract (E) was prepared from rat brain. Microtubules were assembled and centrifuged (7), yielding a supernate (S) and microtubule pellet (P), which was resuspended to volume. The samples were subjected to NaDodSO<sub>4</sub> gel electrophoresis (4% polyacrylamide), transferred to nitrocellulose, and allowed to react with antibodies indicated. Anti-MAP 1B was MAP 1B-4. Arrowheads denote top and dye front of gel.

further, immunoblot analysis of whole brain tissue was performed (Fig. 4a). Surprisingly, MAP 1B was found to be abundant in gray matter as well as in white matter, appearing to be even more abundant in gray matter.

To determine the basis for the apparent discrepancy of this result with the yield of MAP 1B in purified gray and white matter microtubules (Fig. 1), the fate of the MAPs during the initial steps of microtubule purification from rat brain was followed (Fig. 4b). MAP 1A and MAP 2 were removed almost completely from the brain cytosolic extract when microtubules were assembled and sedimented. However, much of the MAP 1B in the extract remained soluble during this procedure, and only a fraction cosedimented with microtubules. The protein was also found to cosediment less efficiently with gray matter than white matter microtubules (data not shown). This accounted for the relative paucity of MAP 1B in gray matter microtubule preparations. In addition, these results indicated that MAP 1B must be much more abundant in both gray and white matter tissue than is revealed by microtubule purification (Fig. 1).

**Distribution of MAP 1B in Brain Tissue and Cultured Brain Cells.** To analyze the cellular and subcellular distribution of

MAP 1B in more detail, immunofluorescence microscopy of rat brain sections and primary brain cell cultures was performed. Although differences in staining intensity were observed among the four anti-MAP 1B antibodies, all of the antibodies stained both white matter and gray, similar to MAP 1A (5), and in sharp contrast to MAP 2 (5, 7-10).

Specific staining of axonal processes and glial cells, as well as of neuronal cell bodies and dendrites, was observed throughout the central nervous system. Fig. 5 shows the pattern of immunoreactivity observed with antibody MAP 1B-4 in the cerebellum. Moderate staining of the Purkinje cell dendrites was observed. In contrast to results obtained with other antibodies to the HMW MAPs, strong staining of parallel fiber axons was seen. Staining of axonal processes in the cerebellar white matter was also prominent.

Immunofluorescence microscopy of primary brain cell cultures was also performed to characterize the subcellular localization of MAP 1B. Flat cells in the culture reacted with the anti-MAP 1B antibodies with a range of intensities.

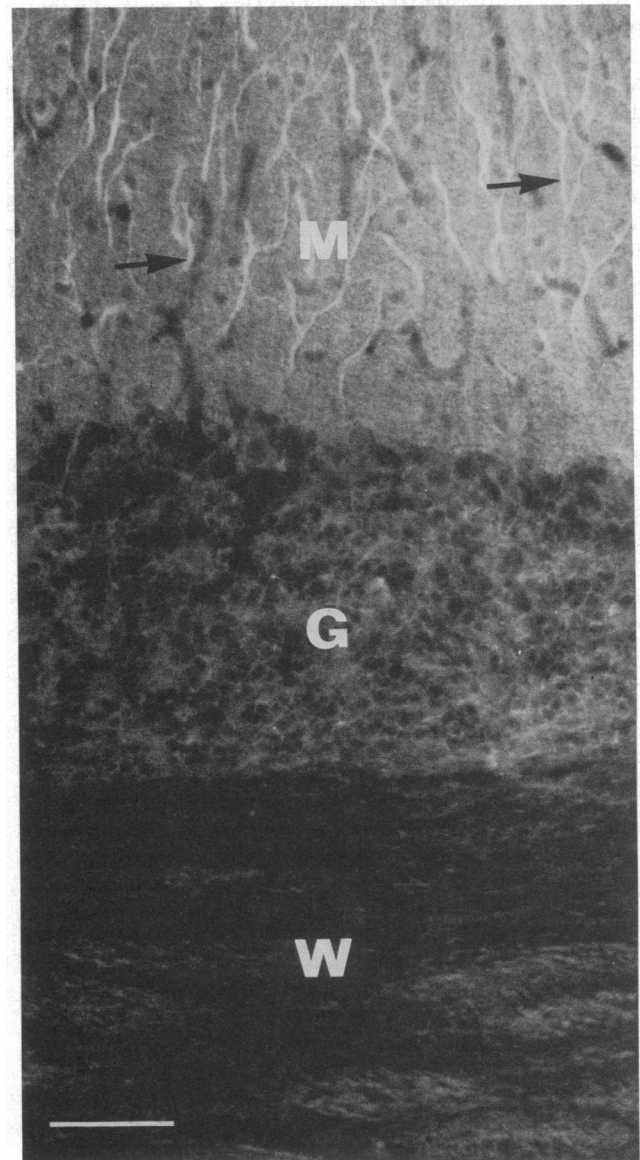


FIG. 5. Immunofluorescence microscopy of MAP 1B in rat cerebellum. A sagittal section of adult rat cerebellum is shown stained with antibody MAP 1B-4. M, molecular layer; G, granule cell layer; W, white matter. Arrows point to Purkinje cell dendrites. (Bar = 50  $\mu$ m.)

Fibrous cytoplasmic staining could be readily observed in many cells (32). The fibers were identified as microtubules by virtue of their sensitivity to colchicine and vinblastine, their reorganization by taxol, and their presence in the mitotic spindle. Some of the more brightly staining cells were positive for myelin basic protein and others were positive for glial fibrillary acidic protein, identifying them as oligodendrocytes and astrocytes, respectively (data not shown; see ref. 5). Most of the brightly staining cells, however, were of neuronal morphology and were positive for MAP 2 (Fig. 6*a*), further characterizing them as neurons. Anti-MAP 2 stained a number of relatively thick, short processes that emanated from these cells. The antibodies to MAP 1B (Fig. 6*b*) stained the same processes and, in addition, reacted intensely with a system of extremely long (up to several millimeters), fine, axon-like processes that failed to react with anti-MAP 2.

### DISCUSSION

In an earlier study, we reported that MAP 1 can be resolved into three electrophoretic bands, of which MAP 1A was the largest and apparently the most abundant (5). We could not conclude whether the other MAP 1 polypeptides represented variants of MAP 1A or distinct protein species. Based on the

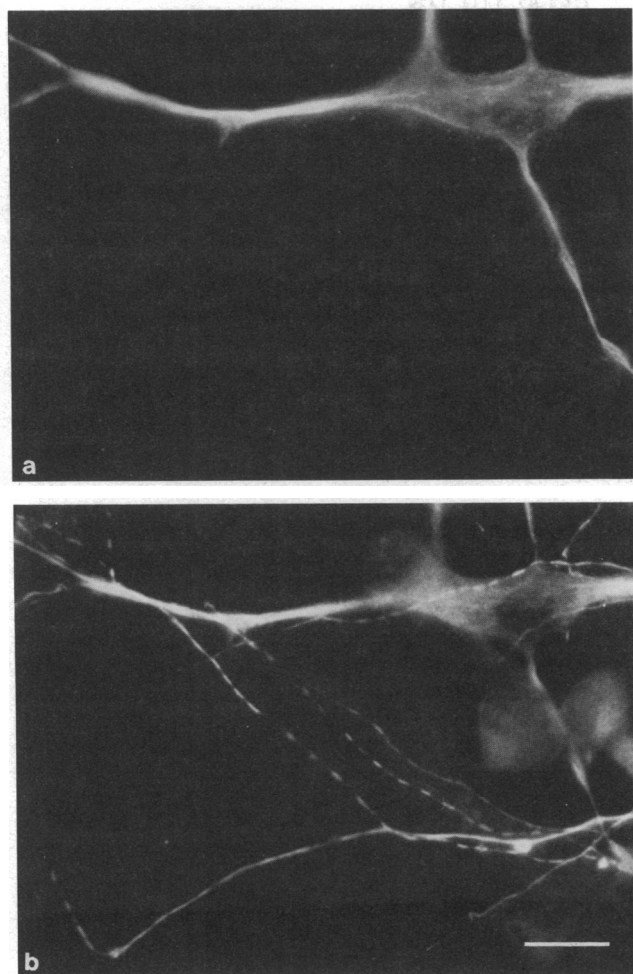


FIG. 6. Double immunofluorescence microscopy of MAP 1B and MAP 2 in primary neuronal cells. Cells were allowed to react with polyclonal anti-MAP 2 (*a*) and monoclonal anti-MAP 1B (*b*, clone MAP 1B-4). Discontinuous staining of fine processes in *b* corresponds to varicosities observed in phase-contrast microscopy. (Bar = 10  $\mu$ m.)

data presented here, we now conclude that MAP 1B represents an abundant and unique protein substantially different in structure from MAP 1A. Whether the two proteins are totally distinct in sequence must await further work. Nonetheless, it is clear that MAP 1B is not simply a degradation product or otherwise modified form of MAP 1A.

Immunoblot analysis indicated the presence of MAP 1B both in whole brain tissue as well as in isolated microtubules (Fig. 4). Yet this protein had not been identified in a decade of intensive analysis of brain microtubules. This appears to be due to the relatively low yield of the protein in microtubules prepared from gray matter (Figs. 1 and 4), the standard tissue source.<sup>‡</sup> In view of the molecular heterogeneity of MAP 1 (ref. 5; this study), immunocytochemical studies involving MAP 1 must be treated with particular caution. This is especially important in view of several recent reports of the unusual reactivity of MAP 1 antibodies with a variety of cellular organelles (21–25, 32).

Curiously, the protein was actually somewhat more abundant in gray matter than in white matter (Fig. 4*a*) despite its virtual absence in purified gray matter microtubules (Fig. 1). This was found to be due to a unique property of the protein among the MAPs characterized in our laboratory (Fig. 4*b*; ref. 26)—namely, its inefficient copurification with tubulin in the earliest stage of preparation. The basis for this effect is uncertain. In a preliminary experiment, it was found that addition of excess tubulin to the brain cytosolic extract following the first microtubule sedimentation step resulted in the sedimentation of additional MAP 1B (unpublished results). This suggests that the inefficiency of binding could be due to a limit in the number of MAP 1B binding sites on the assembled microtubules rather than a loss of the microtubule binding activity of the protein during purification.

Several pieces of evidence indicate that, despite the inefficiency of binding to microtubules in the initial purification step (Fig. 4*b*), MAP 1B is a *bona fide* MAP. (i) That fraction of MAP 1B that does appear in the first microtubule pellet was found to cosediment efficiently with microtubules during subsequent purification steps (18). (ii) Immunofluorescence microscopy of cultured primary brain cells and cultured pituitary cells showed that the protein was associated with microtubules in the cell (refs. 18, 27, 32). (iii) In axons from rats treated with  $\beta,\beta'$ -iminodipropionitrile, which causes segregation of microtubules and neurofilaments within the axoplasm, MAP 1B immunoreactivity cosegregated with the microtubules (28).

Why the protein copurified more efficiently with white matter than gray matter microtubules (Fig. 1) despite the similar abundance in the two brain regions (Fig. 4) is not certain. One possible explanation for this is the higher concentration of MAP 2 in gray matter microtubules, which could more effectively displace MAP 1B from its potential microtubule binding sites.

In both preparations, analysis of purified microtubules results in a severe underestimate of the abundance of MAP 1B in brain tissue. For this reason, we believe this protein to be among the most abundant MAPs, and possibly the most abundant, in the neuronal cell. MAP 1B may be even more abundant than MAP 1A in axonal processes, in particular. This was indicated by the prominent staining of axons by the anti-MAP 1B antibodies (Figs. 5 and 6) compared to anti-MAP 1A, which showed significant, though weaker, axonal staining (5).

Apparent axonal staining in primary cultures of rat brain cells was particularly striking. Though we cannot be certain of the identity of the long, varicose processes that were

<sup>‡</sup>A similar protein may have been identified by Greene *et al.* (20) in PC 12 cells, though that protein could equally well correspond to what we have termed MAP 1C (5).

immunoreactive with anti-MAP 1B antibodies (Fig. 6), we believe they represent axons for two reasons. (i) They are unreactive with anti-MAP 2, which has been found to recognize dendrites almost exclusively in brain tissue (5, 7–10) and in primary neuronal cell culture (29). (ii) The extreme length and fine caliber of the anti-MAP 1B reactive processes is indicative of axonal character, in contrast to the thicker, branched anti-MAP 2 reactive processes. Anti-MAP 1A stained the long processes, but less intensely than anti-MAP 1B (not shown), again suggesting the greater abundance of MAP 1B.

Recent results have indicated that axonal microtubules are embedded in an extensive network of associated fine fibers, in which, in turn, membranous organelles are embedded (28, 30, 31). We propose that MAP 1A and, in particular, MAP 1B, are prominent, and probably the major, components of this axonal "cytomatrix." In this context, the biochemical behavior of MAP 1B (Figs. 1 and 4) suggests a unique mode of interaction with microtubules. Perhaps only a fraction of MAP 1B in the axon is directly associated with the microtubule, while the remainder forms the microtubule-associated matrix. Further work will clearly be needed to determine the precise role of MAP 1B in the construction of both the axonal and the dendritic cytoskeleton and in the movement of membranous organelles through the neuronal process.

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