Association of Microtubule-associated Protein 2 (MAP 2) with Microtubules and Intermediate Filaments in Cultured Brain Cells

GEORGE S. BLOOM and RICHARD B. VALLEE
Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

ABSTRACT The classification of MAP 2 as a microtubule-associated protein is based on its affinity for microtubules in vitro and its filamentous distribution in cultured cells. We sought to determine whether MAP 2 is also able to bind in situ to organelles other than microtubules. For this purpose, primary cultures of rat brain cells were stained for immunofluorescence microscopy with a rabbit anti-MAP 2 antibody prepared in our laboratory, as well as with antibodies to vimentin, an intermediate filament protein, and to tubulin, the major subunit of microtubules. MAP 2 was present on cytoplasmic fibers in neurons and in a subpopulation of the flat cells present in the cultures. Our observations were concentrated on the flat cells because of their suitability for high-resolution immunofluorescence microscopy. Double antibody staining revealed co-localization of MAP 2 with both tubulin and vimentin in the flat cells. Pretreatment of the cultures with vinblastine resulted in the redistribution of MAP 2 into perinuclear cables that contained vimentin. Tubulin paracrystals were not stained by anti-MAP 2. In cells extracted with digitonin, the normal fibrillar distribution of MAP 2 was resistant to several treatments (PIPES buffer plus 10 mM Ca²⁺, phosphate buffer at pH 7 or 9) that induced depolymerization of microtubules, but not intermediate filaments. Staining of the primary brain cells was not observed with preimmune serum nor with immune serum adsorbed prior to use with pure MAP 2. We detected MAP 2 on intermediate filaments not only with anti-MAP 2 serum, but also with affinity purified anti-MAP 2 and with a monoclonal anti-MAP 2 prepared in another laboratory. We conclude from these experiments that material recognized by anti-MAP 2 antibodies associates with both microtubules and intermediate filaments. We propose that one function of MAP 2 is to cross-link the two types of cellular filaments.

Since the introduction of procedures for purifying cytoplasmic microtubules (6, 48), it has become evident that these structures are composed of numerous polypeptide species. Tubulin is the predominant component of cytoplasmic microtubules. In addition, numerous proteins that appear to be associated with tubulin have been discovered. These microtubule-associated proteins, or MAPs (53), have been identified on the basis of their affinity for microtubules in vitro (4, 12, 38, 53), and on the basis of immunocytochemical data (10, 11, 13, 14, 27, 31, 41, 50, 51, 52). The MAPs have been implicated in controlling microtubule assembly (37, 63). In addition, at least two MAP species, MAP 2 (25, 30) and MAP 1 (61), represent fine filamentous projections regularly spaced on the microtubule surface. This has given rise to speculation that these proteins may mediate the interaction of microtubules with other organelles and may, therefore, be important in microtubule function as well as microtubule assembly. Evidence for the binding of MAPs in vitro to a variety of structures in addition to microtubules has been reported (23, 34, 45, 46, 49, 54), further supporting a role for MAPs in the interaction of microtubules with other cellular components.

MAP 2 has been extensively characterized. It is the most abundant MAP species identified in brain tissue, its major source. This laboratory has shown that the protein may be divided into two structural domains (58). One domain contains the microtubule binding site and promotes microtubule assembly. The other, larger domain represents the portion of MAP 2 observed as a projection on the microtubule surface. The projection domain contains a binding site for the regulatory subunit of a type II cAMP-dependent protein kinase, which...
phosphorylates MAP 2 (55, 60). Presumably, the projection domain also contains a site or sites for binding of other cytoplasmic organelles.

Evidence for the binding of MAP 2 in vitro to actin filaments, coated vesicles, and intermediate filaments has been reported (1, 24, 34, 40, 45, 46). While these studies have identified structures that are capable of interacting with MAP 2 in vitro, they leave open the question of which organelles actually bind MAP 2 in cells. We initiated our study to resolve this question and to further characterize the binding domains of this protein. We reasoned that if the cellular microtubule binding site for MAP 2 was dissolved it might be possible to identify other organelles to which the MAP bound. We chose to work with primary cultures of brain cells because these cells contain MAP 2 in apparently high concentration and include a population of well-spread cells that are suitable for high-resolution immunocytochemical techniques (27, 41). We report here the results of a study to determine which organelles other than microtubules bound to MAP 2. Whole brains from rats varying in age from 16-d fetuses to 1- (8, 66, 67) were adapted to produce cultures with optimal survival of cells found in this protein. We reasoned that if the cellular microtubule binding site for MAP 2 was dissolved it might be possible to identify other organelles to which the MAP bound. We chose to work with primary cultures of brain cells because these cells contain MAP 2 in apparently high concentration and include a population of well-spread cells that are suitable for high-resolution immunocytochemical techniques (27, 41). We report here the results of a study to determine which organelles other than microtubules bound to MAP 2.

**MATERIALS AND METHODS**

**Anti-MAP 2 Antibodies:** We prepared a rabbit antibody to calf brain MAP 2 in this laboratory, and used it to immunoprecipitate the MAP 2 protein kinase complex (55) and to localize MAP 2 by immunofluorescence in the rat central nervous system (35) (Miller, P., W. E. Theurkauf, R. B. Vallee, and P. De Camilli, manuscript in preparation). The MAP 2 immunogen was purified by a three-step process designed to insure antigen purity. First, calf brain microtubules were purified by four cycles of assembly and disassembly in the absence of glycerol (60). The microtubules were exposed to elevated temperatures (90–100°C) to coagulate tubulin, MAP 1, and a variety of minor polypeptides (18, 25, 30). The MAP 2-enriched soluble fraction was finally subjected to preparative SDS PAGE. After staining with Coomassie Brilliant Blue R250, the MAP 2 doublet (30) was excised from the gel and homogenized with Freund’s complete adjuvant. A rabbit was injected subcutaneously with ~100 μg of MAP 2 on each of three occasions, and was bled 1 wk after the final injection.

Affinity-purified anti-MAP 2 was prepared by passage of the immune serum over a MAP 2-Sepharose 4B column. The MAP 2 used here was prepared from a population of well-spread cells that are suitable for high-resolution immunocytochemical techniques (27, 41). We report here the results of a study to determine which organelles other than microtubules bound to MAP 2.

**RESULTS**

**Characterization of Anti-MAP 2**

Partial characterization of our anti-MAP 2 antibody has been reported elsewhere (35; Miller, P., W. E. Theurkauf, R. B. Vallee, and P. De Camilli, manuscript in preparation). The antibody stained neurons in sections of mature rat brain, and showed particularly bright staining of neuronal dendrites. The
antibody specifically stained a protein of the molecular weight of MAP 2 in whole brain, purified microtubules, and purified preparations of MAP 2. For the present study, we examined newborn rat brain tissue by immunocytochemical means (Fig. 1). It may be observed that only a band at the position of MAP 2 reacts with the anti-MAP 2 antiserum.

**Immunofluorescent Detection of MAP 2 in Primary Cultures of Rat Brain Cells**

The primary cultures of rat brain cells used in this study contained a variety of morphologically distinct cells. When these cultures were reacted with anti-MAP 2 antibody, only selected cells were stained. Fig. 2a and b illustrate the most common MAP 2-positive cells. These cells had a neuronal morphology, with small soma (10-25 μm diameter) and numerous fine processes that occasionally extended for >100 μm. The processes frequently exhibited periodic varicosities. In addition to containing cells of neuronal morphology, the cultures also contained an abundance of flat, well-spread cells, some of which stained with anti-MAP 2 (Fig. 2c). Almost all MAP 2-positive neurone-like cells were found to possess tubulin toxin receptors (not shown) and were probably neurons, therefore (16, 36). In addition to containing cells of neuronal morphology, the cultures also contained an abundance of flat, well-spread cells, some of which stained with anti-MAP 2 (Fig. 2c). Almost all MAP 2-positive neurone-like cells were found to possess tubulin toxin receptors (not shown) and were probably neurons, therefore (16, 36).

Co-localization of MAP 2 and Tubulin

The MAP 2-positive flat cells provided an excellent experimental system for high-resolution, immunofluorescent location of MAP 2, which was not possible with the more compact neurons. Therefore, our observations focused on the flat sub-population of cells, as described in the remainder of the paper.

**Uncoupling of MAP 2–Tubulin Co-Localization**

The anti-MAP 2 staining patterns shown in Fig. 3 are characteristic of microtubules. To determine the cellular location of MAP 2 in the absence of microtubules, we employed two approaches. First, the cultures were exposed to vinblastine sulfate for several hours prior to fixation and antibody staining. Antitubulin staining (not shown) revealed the absence of microtubules in the flat cells, and the appearance of brightly staining tubulin paracrystals, as is characteristic of cells exposed to this drug. Thin-section electron microscopy of these cultures similarly revealed the complete absence of microtubules (not shown). Fig. 4 shows an example of a flat cell that showed positive staining with anti-MAP 2. The single, striking paracrystal in this cell was readily detected by phase-contrast microscopy (Fig. 4a). However, the antibody conspicuously failed to stain the paracrystal. Instead, a sinuous cable of bright fluorescence surrounding the nucleus was observed (Fig. 4b). This pattern of staining was clearly not consistent with that found for tubulin. Rather, it was consistent with that expected for certain classes of intermediate filaments. An intermediate filamentlike distribution of MAP 2 was also observed in cells exposed to colchicine (not shown), and was apparent with vinblastine or colchicine incubations ranging from 1 to 24 h. Thus, our results appeared to reveal a second binding site for MAP 2 in cells, one associated with intermediate filaments.

As an independent approach to eliminate microtubules, the primary brain cells were extracted with digitonin under conditions known to result in the disassembly of microtubules. The cells were then fixed and double-stained for tubulin and MAP 2, as shown in Fig. 5. MAP 2 and tubulin co-localized on cytoplasmic fibrils in cells extracted with PIPES buffer (Fig. 5a and b), which preserves microtubules. Both phosphate buffers (Fig. 5f and h) and PIPES buffer with 10 mM Ca++ (Fig. 5d) dissolved microtubules, as judged by antitubulin staining. However, the same cells still exhibited a fibrillar pattern of staining with anti-MAP 2 (Fig. 5c, e, and g), indicating the existence of a nonmicrotubule binding site for MAP 2 in these cells. The fibrillar nature of the staining pattern and its stability in the extracted cells is again consistent with localization of MAP 2 on intermediate filaments.
Comparison of Polyclonal and Monoclonal Anti-MAP 2

To determine whether the results obtained were unique to our anti-MAP 2 antibody preparation, vinblastine-treated cells were double-labeled with our polyclonal rabbit antibody and a monoclonal antibody prepared by Izant and McIntosh (27). The results of this comparison are shown in Fig. 8. It is clear that both antibody preparations stained intermediate filament cables, but not tubulin paracrystals, in the flat primary brain cells.

DISCUSSION

MAP 2 has been classified as a MAP primarily on the basis of its affinity for microtubules in vitro (4, 38, 53). In addition, antibodies to this protein have been found to stain cytoplasmic fibers in a limited number of cultured cell types (27, 31, 41, 57). The conclusion that these fibers were microtubules was based on co-localization of the immunoreactive species with tubulin (27) and abolition of the fibrous staining pattern by antimicrotubule drugs (27, 31). Here we report that in cultured brain cells an anti-MAP 2 antibody stained fibrils that co-localized with both microtubules (see Fig. 3) and intermediate filaments (see Fig. 6). The intermediate filament-like staining pattern persisted after dissolution of microtubules in living cells by antimicrotubule drugs (see Figs. 4, 7, and 8), or in cytoskeletal preparations by appropriate buffers (see Fig. 5). These results indicated that material cross-reactive with MAP 2 may be associated with intermediate filaments, as well as with microtubules.

Specificity of the Anti-MAP 2 Antibody

It is possible that these results simply reflect the presence in our anti-MAP 2 preparation of contaminating antibodies specific for an intermediate filament protein. We consider this quite unlikely since both affinity purified anti-MAP 2 (see Fig. 4b) and an independently prepared monoclonal anti-MAP 2 (see Fig. 8b) stained intermediate filaments. In addition, nei-
FIGURE 3 Co-localization of MAP 2 and tubulin. Flat, MAP 2-positive cells are shown in interphase (a and b), telophase (c and d), and metaphase (e and f). Cells were incubated with rabbit anti-MAP 2 serum plus either goat (b and f) or guinea pig (d) antiserum to tubulin. Primary antibodies were then stained simultaneously with either rhodamine-sheep anti-rabbit IgG plus fluorescein-sheep anti-goat IgG, or with rhodamine-goat anti-rabbit IgG plus fluorescein-goat anti-guinea pig IgG. (a, c, and e) anti-MAP 2; (b, d, and f) antitubulin. Bars, 10 μm. (a and b) X 800. (c and d) X 1,100. (e and f) X 1,200.

FIGURE 4 Effect of vinblastine on MAP 2 staining. A primary brain cell culture was treated with 10 μM vinblastine sulfate for 15 h before being stained with affinity purified anti-MAP 2 and rhodamine-sheep anti-rabbit IgG. (a) Phase contrast. (b) Anti-MAP 2. Immediately above the nucleus is a prominent tubulin paracrystal easily visualized by phase contrast, but not stained by the antibody. Bar, 10 μm. X 1,300.

ther immune serum adsorbed prior to use with pure MAP 2, nor preimmune serum-stained cytoplasmic filaments.

This indicates that the intermediate filament-associated antigen recognized by anti-MAP 2 is truly cross-reactive with MAP 2. Nonetheless, it is possible that this antigen represents a protein quite distinct from MAP 2, but which is immunologically cross-reactive due to limited amino acid sequence homology. Several examples of distinct, but immunologically cross-reactive cytoskeletal proteins have been reported (3, 5, 39, 42). Indeed, it has been found that MAP 2 itself reacts with an antispectrin antibody (15).

Thus, it is possible that we have identified an intermediate filament protein which shares one or more antigenic sites with MAP 2, but is otherwise distinct. However, several considerations lead us to believe that MAP 2 itself is the antigen associated with intermediate filaments. First, the only antigenic species detected by our antibody in a mixture of total newborn rat brain proteins has a mobility in SDS PAGE identical to that of MAP 2 (see Fig. 1). Second, the antibody stained only a limited number of the flat cells in these cultures (10% or less). Among these cells, unambiguous staining of microtubules was observed during mitosis, while intermediate filaments were clearly stained after vinblastine treatment. Thus, if two distinct, cross-reactive proteins exist in primary brain cells, both would be present in the same small subpopulation of flat cells. While such a coincidence is conceivable, it seems highly unlikely.

Finally, we also observed co-localization of MAP 2 with both
FIGURE 5 Presence of MAP 2 on cytoplasmic fibers in isolated cytoskeletons lacking microtubules. Cells were extracted with digitonin as described in Materials and Methods, incubated for 15 min at room temperature in various buffers, fixed, and treated with rabbit anti-MAP 2 serum and guinea pig antitubulin, followed by rhodamine goat anti-rabbit IgG and fluorescein goat anti-guinea pig IgG. (a, c, e, and g) Anti-MAP 2 staining; (b, d, f, and h) Antitubulin staining. (a and b) control buffer: 100 mM PIPES, pH 6.6, 1 mM MgSO4 and 1 mM EGTA; (c and d), the same, plus 10 mM CaCl2; (e and f) 100 mM phosphate buffer at pH 7; (g and h), 100 mM phosphate buffer at pH 9. Bar, 10 μm. × 700.

Intracellular Functions of MAP 2

Immunocytochemical, electron microscopic, and physiological evidence (2, 17, 21, 26, 33, 44, 47) have pointed to an association between microtubules and intermediate filaments. In particular, double immunofluorescence microscopy has revealed extensive co-localization of microtubules with desmin filaments in chicken gizzard cells (21), with vimentin filaments in fibroblasts (2) and with glial filaments in primary astrocytes (G. S. Bloom, R. B. Vallee, and R. Liem, unpublished observations). These findings suggest that microtubules and intermediate filaments may be commonly connected to one another in cells, presumably by cross-linking proteins.

Recently, Wiche and co-workers (43, 64) proposed that a high molecular weight MAP-like protein cross-links microtubules and intermediate filaments in numerous varieties of microtubules and intermediate filaments in the B104 neuroblastoma cell line. In this homogeneous population of cells, only a single protein of Mr 270,000 reacted with anti-MAP 2, further supporting our contention that a single protein species binds to both microtubules and intermediate filaments (G. S. Bloom and R. B. Vallee, unpublished observations).

Although other laboratories have used anti-MAP 2 antibodies for immunofluorescence microscopy on cultured cells (27, 31, 41, 57), the association of MAP 2 with intermediate filaments has not been reported until now. Detection of this association may depend on the use of an appropriate combination of cultured cell system and experimental protocols. Indeed, we demonstrate here that a monoclonal anti-MAP 2 antibody (27) independently prepared in another laboratory can stain intermediate filaments in our vinblastine-treated primary brain cells (see Fig. 8).
cultured cells. However, subsequent studies in the same laboratory revealed that the protein, now known as "plectin," has an intracellular distribution that is markedly distinct from microtubules and intermediate filaments (65). Therefore, we feel it is unlikely that plectin acts as a cross-linker. In contrast, our finding that MAP 2 can co-localize with both microtubules and intermediate filaments indicates that this protein, in particular, is responsible for the observed interactions of the two classes of cytoplasmic fibers. Since MAP 2 may not be universally distributed in cells (27, 41, 57), it is possible that other unidentified proteins also serve this function.

Although MAP 2 was found to be co-distributed with vimentin, we do not know whether vimentin is the only intermediate filament protein in the MAP 2-positive, flat brain cells. For this reason, we are not yet certain whether MAP 2 binds directly to vimentin itself, or to another intermediate filament protein with a similar intracellular distribution. Further biochemical work will be required to determine the specific intermediate filament proteins that are able to bind to MAP 2, and to define the relevant binding domains on MAP 2 (cf. reference 58).

Our data represent the first demonstration that one role for MAP 2 in cells is to mediate interactions of microtubules and intermediate filaments. Whether this is the only cellular function of MAP 2 is not clear, since the protein is most abundant in vivo in neuronal dendrites (35; Miller, P., W. E. Theurkauf, R. B. Vallee, and P. De Camilli, manuscript in preparation), which contain abundant microtubules, but few intermediate filaments.

**Figure 6** Co-localization of MAP 2 and vimentin. Cells were stained with rabbit anti-MAP 2 serum and monoclonal mouse antivimentin, followed by rhodamine goat anti-rabbit IgG and fluorescein-goat antirabbit IgG. (a) Anti-MAP 2 staining; (b) Antivimentin staining. Bar, 10 μm. X 900.

**Figure 7** Co-localization of MAP 2 and vimentin on perinuclear cables in vinblastine-treated cells. Cells were treated for 17 h with 10 μM vinblastine sulfate. They were then stained with rabbit antiserum to MAP 2 and monoclonal mouse antivimentin, followed by rhodamine goat anti-rabbit IgG and fluorescein goat anti-mouse IgG. (a) Phase contrast; (b) Anti-MAP 2; (c) Antivimentin. Bar, 10 μm. X 800.

**Figure 8** Detection of MAP 2 on intermediate-filament cables in vinblastine-treated cells by polyclonal and monoclonal antibodies. Cells were treated for 18 h with 10 μM vinblastine sulfate before being stained with rabbit anti-MAP 2 serum and monoclonal mouse anti-MAP 2, followed by a combination of goat anti-rabbit IgG and fluorescein goat anti-mouse IgG. (a) Phase contrast; (b) Rabbit anti-MAP 2 serum; (c) Monoclonal mouse anti-MAP 2 antibody. Bar, 10 μm. X 900.
filaments. We suggest, therefore, that cross-linking of microtubules and intermediate filaments may be only one role of the protein, and that other cellular functions remain to be defined. Recent work by Izant and co-workers (28, 29) has suggested that the 210,000-dalton MAP identified in HeLa cells (9, 62) may be heterogeneous with regard to cellular distribution and function. Similarly, MAP 2 may be a complex protein species, individual components of which bind to a variety of cellular organelles. Indeed, the size heterogeneity of MAP 2 in SDS PAGE (30) may signify functional heterogeneity as well. It is also possible that the binding specificity of MAP 2 is regulated by posttranslational modification. For example, phosphorylation of MAP 2 by its associated cAMP-dependent protein kinase (55, 60) or by other protein kinases could potentially regulate whether MAP 2 binds to intermediate filaments or to other structures.

We would like to thank Jonathan Izant and Richard McIntosh for the monoclonal mouse anti-MAP 2, Robert Weising for the goat antitubulin, and William Hág for the goat antitoxin. We also express our thanks to Francis Land for his technical assistance.

This work was supported by National Institutes of Health research grant GM-26701 to Richard B. Valee.

Received for publication 9 November 1982, and in revised form 4 February 1983.

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