

# Microtubule-Associated Proteins: Subunits of the Cytomatrix

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## *Microtubule-Associated Proteins of Brain Tissue*

Microtubules isolated from brain tissue are composed of tubulin and a variety of nontubulin proteins known as microtubule-associated proteins (MAPs).<sup>1</sup> The most prominent MAPs in brain microtubule preparations are known as the high molecular weight MAPs (8, 50) because of their large subunit size (~300,000 daltons). Brain microtubules also contain a smaller amount of a group of proteins of  $M_r$  55,000–62,000 known as the tau MAPs (10, 63), as well as a number of minor protein species. However, because of the relative abundance and interesting morphology of the high molecular weight MAPs, these have been the most intensely studied of all of the known MAPs.

The high molecular weight MAPs appear as fine filamentous arms on the surface of purified microtubules when examined by thin-section electron microscopy (13, 34), rapid-freeze, deep-etch electron microscopy (21), or metal shadowing (61, 66). Their appearance suggests that these proteins play a role in mediating the interaction of microtubules with other cellular organelles. As extensively discussed in this supplement, a considerable body of evidence points to the existence of a network of interconnections between the various structural components of the cell, including microtubules. In investigating the high molecular weight brain MAPs, therefore, we are presumably examining isolated components of this "cytomatrix." It is our hope that this line of investigation will eventually contribute to an understanding of how the cytomatrix functions and, perhaps of even greater interest ultimately, how its behavior is modulated.

The high molecular weight brain MAPs have an additional property that has stimulated considerable research effort in a number of laboratories, that of promoting the polymerization of tubulin to form microtubules. Thus, these proteins may be multifunctional, increasing the inherent interest in their isolation and characterization.

<sup>1</sup> *Abbreviation used in this paper:* MAP, microtubule-associated protein.

## BIOCHEMICAL AND STRUCTURAL PROPERTIES OF MAP 1 AND MAP 2

On high-percent polyacrylamide gels the high molecular weight MAPs appear as two electrophoretic species known as MAP 1 and MAP 2 (Fig. 1A) (50). As discussed in the next paragraph, this distinction oversimplifies the true complexity of these proteins. Nevertheless, it has been useful and has some basis in the biochemical properties of the proteins. MAP 1 and MAP 2 have been separated into distinct fractions (20, 25, 58). Each fraction has been found to contain a single high molecular weight electrophoretic species when examined with high-percent polyacrylamide gels, but multiple species when examined on more highly resolving electrophoretic systems (see below). When recombined and assembled with tubulin, both MAP 1 (58) and MAP 2 (20, 25) appeared as projecting arms on the microtubule surface. In addition, both MAP 1 (27, 58) and MAP 2 (20, 25, 35) promoted microtubule assembly. Thus, MAP 1 and MAP 2 clearly have important features in common.

Fig. 1B shows a low-percent polyacrylamide gel revealing the complexity of the high molecular weight MAPs. Two MAP 2 species, which we call MAP 2A and MAP 2B, can be distinguished from the MAP 1 polypeptides on the basis of their heat stability (6, 20, 25), a remarkable property for proteins of this size, cochromatography (57), and immunological cross-reactivity (3, 60). MAP 1 consists of at least three high molecular weight bands that we call MAP 1A, MAP 1B, and MAP 1C (6). None of these proteins is heat stable, and none has been found to cross-react with MAP 2 or with each other (6 and Bloom, G. S., F. C. Luca, and R. B. Vallee, unpublished results). The latter observation raises the possibility that, whereas MAP 2A and MAP 2B may be representatives of a very closely related class of proteins, the MAP 1 polypeptides are diverse in their properties and, perhaps, in their functions.

We have observed that exposure of the high molecular weight MAPs to chymotrypsin or trypsin results in the disappearance of the MAP arms from the microtubule surface

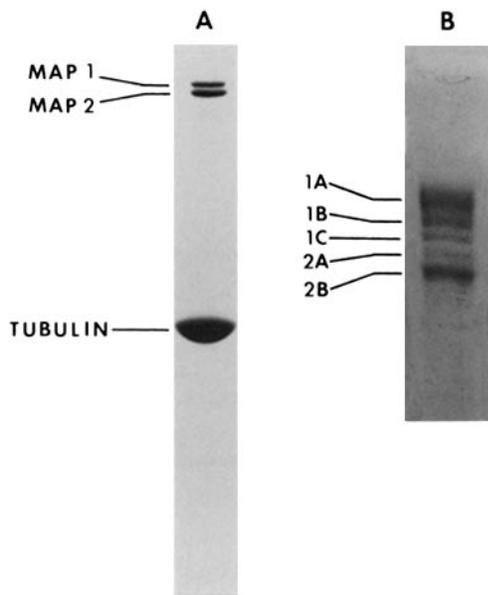


FIGURE 1 Electrophoretic components of microtubules. (A) 9% polyacrylamide gel of microtubules prepared using taxol from calf brain cerebral cortex, showing tubulin and two high molecular weight MAP bands. From Vallee (55). (B) 4% polyacrylamide gel of microtubules prepared using taxol from calf brain white matter. Tubulin and other minor components ran with the dye front. Only the high molecular weight portion of the gel is shown. From Bloom et al. (6).

(54, 56). MAP 1A, MAP 1B, MAP 2A, and MAP 2B all showed extreme sensitivity to proteases, whereas MAP 1C was relatively resistant, suggesting that MAP 1C is structurally quite different from the other MAPs (6). Analysis of the fragments of MAP 2 generated by chymotrypsin revealed that most of the MAP 2 polypeptide chain (~235,000 daltons) was part of the filamentous arm observed projecting from the microtubule surface (54) (Fig. 2). The remainder of the molecule contained the microtubule-binding domain. This portion of the molecule had the interesting property of promoting microtubule assembly, apparently to the same extent as intact MAP 2.

We have found that both MAP 1 and MAP 2 contain low molecular weight polypeptide components. Associated with MAP 2 at low molar stoichiometry are polypeptides of  $M_r$  70,000, 54,000, and 39,000 (57). Although we do not know the identity of the  $M_r$  70,000 species, we have found that the latter two species represent, respectively, the regulatory and catalytic subunits of a type-II cyclic AMP-dependent protein kinase that is associated with the arm portion of the MAP 2 molecule (52, 57) (Fig. 3). Remarkably, the MAP-associated enzyme represented 30–35% of the total cyclic AMP-dependent protein kinase in brain cytosol, one indication that MAP 2 phosphorylation may be a rather significant phosphorylation reaction in brain tissue. MAP 2 was the primary substrate for phosphorylation by its associated kinase in purified microtubule preparations and was also a prominent substrate in whole-brain cytosol. Inorganic phosphate analysis of MAP 2 as isolated revealed from 8 to 13 phosphate residues per molecule (53). The MAP 2-associated kinase rapidly catalyzed *in vitro* a further increase in phosphate content to a level of 20 to 22 residues per molecule. Both the assembly-promoting domain of MAP 2 and the arm domain were phosphorylated *in vitro* (54) (Fig. 2). This raises the possibility

that phosphorylation of MAP 2 may regulate the integrity of the cytomatrix either by modulating the interaction of the MAP with the microtubule itself or with some other cytoplasmic component.

The low molecular weight components of MAP 1 (58), which we have termed light chains 1 and 2 ( $M_r$  30,000 and 28,000), differed from those of MAP 2. The MAP 1 light chains were associated with the high molecular weight MAP 1 polypeptides in a nearly one-to-one molar stoichiometry. We do not yet know whether the light chains are structural or enzymatic components of MAP 1, or with which high molecular weight polypeptide they are associated.

#### DIFFERENTIAL CELLULAR DISTRIBUTION OF MAP 1 AND MAP 2

Despite a decade of work on the high molecular weight MAPs, the cellular origin of these proteins has only recently become known. Two reports (24, 40) indicated that MAP 2 was undetectable by immunofluorescence microscopy in a wide variety of cultured cell lines, including lines of neuronal origin. Nonetheless, antibodies prepared against the entire complement of high molecular weight MAPs did stain microtubules in cultured cells of both neuronal and nonneuronal origin (11, 47, 48). To complicate matters further, biochemi-

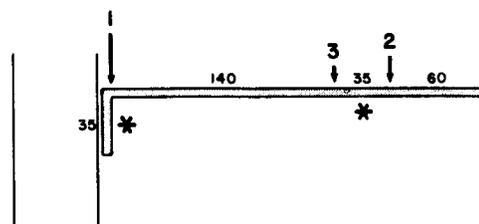


FIGURE 2 Schematic representation of MAP 2 digestion by chymotrypsin. The MAP 2 polypeptide chain is shown bound to a microtubule represented by two vertical lines at left. Attack at site indicated by arrow 1 releases the arm portion of MAP 2, leaving the 35,000-dalton microtubule-binding and assembly-promoting domain bound to the microtubule. Less rapid cleavage occurs at sites 2 and 3. Asterisks indicate phosphopeptides. From Vallee (54).

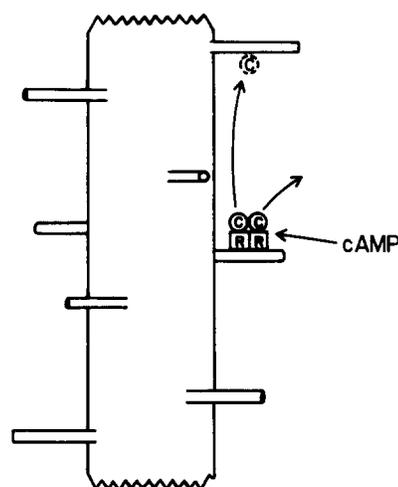


FIGURE 3 The MAP 2 protein kinase complex. A cAMP-dependent protein kinase molecule is shown associated via its regulatory subunits (R) with the arm portion of MAP 2. Binding of cAMP to the regulatory subunits releases the catalytic subunits of the enzyme (C), which are now activated to phosphorylate other MAP 2 molecules. From Theurkauf and Vallee (52).

cal preparations of MAPs from a variety of cultured cells generally lacked significant amounts of the high molecular weight MAPs identified in brain tissue (9, 14, 15, 36, 38, 62).

Recently, Matus et al. (31) stained sections of brain tissue with a polyspecific antibody prepared against the entire complement of high molecular weight MAPs to determine the distribution of these proteins within the brain. Only neurons were stained by their antibody preparation. Furthermore, within neurons, only the dendritic processes and cell bodies were immunoreactive. Axonal processes and glial cells were not stained.

Taken together, these studies have left the question of the distribution of the high molecular weight MAPs in a confusing state. We have used two approaches to resolve this problem. On the one hand, we have prepared antibodies specific for the respective high molecular weight MAPs and used these for immunofluorescence microscopy of both cultured cells and tissue sections. We have complemented this approach with the biochemical isolation of microtubules from different brain regions.

Using a new procedure for purifying microtubules that depends on the assembly-promoting activity of taxol (55), we compared the composition of MAPs in brain gray matter and white matter (Fig. 4). Although both areas of brain tissue contain glial cells and axons, the comparison of the two areas was of interest because only gray matter contains substantial numbers of neuronal cell bodies and dendrites. The ratio of MAP 1 to tubulin in microtubules purified from gray and white matter was the same, whereas MAP 2 was greatly enriched in gray matter microtubules. This suggested that MAP 1 is widespread within brain tissue, perhaps occurring in axonal processes or glial cells or both, while MAP 2 is restricted in its distribution.

Staining of brain sections with a polyclonal rabbit anti-MAP 2 antibody revealed that MAP 2 was restricted to neurons (12, 32). Within neurons, staining was only found in cell bodies and dendritic processes, with axonal staining being undetectable. This result was consistent with the biochemical detection of MAP 2 at much higher levels in gray matter than in white (Fig. 4), though the same data indicated that low levels of MAP 2, apparently undetectable by immunofluorescence microscopy, might be present in axons or glial cells.

In contrast to these results, a monoclonal antibody to MAP 1A, the most prominent component of the MAP 1 polypeptide complex (see Fig. 1), stained axons as well as dendrites and neuronal cell bodies (6). Glial cells were also intensely stained by the antibody, indicating that MAP 1A was present in more than one cell type. Further analysis revealed that

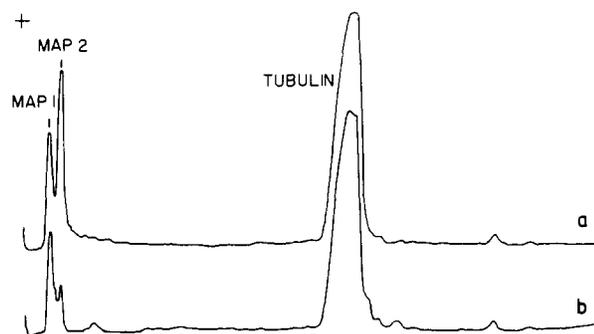


FIGURE 4 Densitometric scans of SDS polyacrylamide gel lanes showing microtubules purified from calf brain gray matter (a) and white matter (b). From Vallee (55).

MAP 1A was extensively distributed among a wide variety of cell types and was prominent both in interphase microtubules and in the mitotic spindle of dividing cells (7). Immunoblot analysis indicated that an immunoreactive polypeptide at the electrophoretic position of MAP 1A could be detected in all cell types examined.

Thus, the two high molecular weight MAPs have quite different distributions. MAP 2 is highly restricted in its distribution, whereas at least the major component of the MAP 1 complex of polypeptides, MAP 1A, is widespread. These findings appear to reconcile some of the contradictory results of the earlier studies cited above but leave open the question of why MAP 1A was not detected in the biochemical studies. The great sensitivity of MAP 1A to proteolysis (6, 56; see above) suggests that the protein may have been destroyed under the conditions that were employed in the earlier studies. Our recent work also conflicts with the findings of Matus et al. (31), which imply that all of the high molecular weight MAPs are restricted to the dendritic processes and cell bodies of neurons. We find that it is MAP 2 alone that has this interesting distribution.

#### MAP 2 MEDIATES THE INTERACTION OF TWO FILAMENT SYSTEMS

The appearance of the high molecular weight MAPs as arms on the microtubule surface suggests that these proteins are capable of interacting via their arm domains with other cytoplasmic components. A considerable body of ultrastructural and physiological evidence indicates that microtubules interact with other microtubules, with intermediate filaments, and with membrane-bounded organelles (1, 16, 17, 19, 22, 28, 46). These observations have prompted a number of investigations into the ability of the MAPs to interact with other organelles *in vitro*. These organelles have included neurofilaments (29, 33, 42), secretory granules (49, 51), and coated vesicles (44). In addition, it has been found that MAPs interact with actin filaments *in vitro* (18, 45). It has been difficult to distinguish which of these associations are specific and which are not. Several of these associations are very weak, making quantitative biochemical characterization quite difficult (18, 45, 51). Others are sufficiently strong to allow for biochemical characterization by standard means. However, so far, it has been difficult to show that these interactions are of defined stoichiometry or are specific to particular MAPs.

This situation has prompted us to approach the same question using conditions selected to perturb minimally the natural interactions of MAPs with their binding sites in the cell (4). We reasoned that if we were to dissolve microtubules in living cells with microtubule-disrupting drugs, the MAPs might remain attached to other organelles via binding sites other than the microtubule binding domain (Fig. 2). We might then be able to identify these organelles by immunofluorescence microscopy using antibodies specific for each of the MAPs.

Fig. 5 shows a primary cultured brain cell that had been treated with vinblastine to disassemble microtubules and then was processed for immunofluorescence microscopy using our rabbit polyclonal antibody to MAP 2. Whereas most of the MAP 2-positive cells in the culture had the morphology expected for neurons, some cells, such as the one shown in Fig. 5, were flat and relatively symmetric, and, thus, suitable for high-resolution immunofluorescence microscopy. These cells probably represent undifferentiated neurons (4). It may

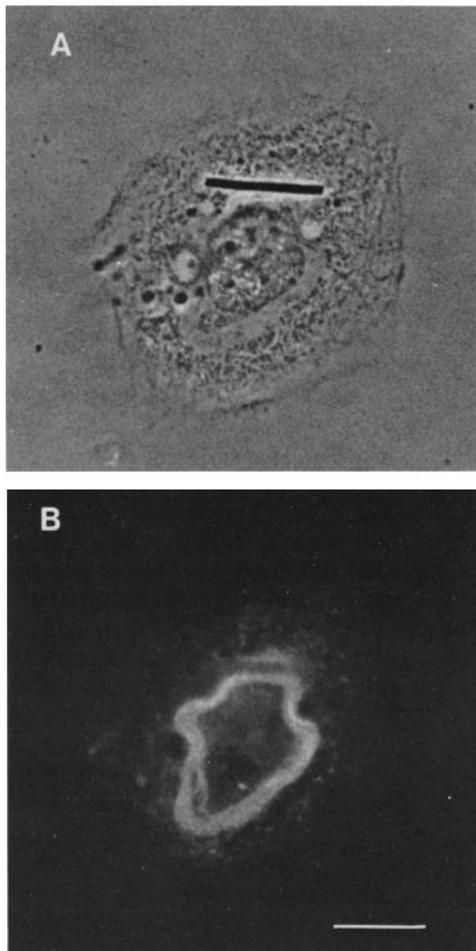


FIGURE 5 Immunofluorescence microscopy of a primary rat brain cell pretreated with vinblastine and stained with a polyclonal rabbit anti-MAP 2 antibody. (A) Phase-contrast micrograph showing phase-dense tubulin paracrystal (dark bar) induced by vinblastine. (B) Fluorescence micrograph showing MAP 2 in perinuclear intermediate filament cable. From Bloom and Vallee (4). Bar, 10  $\mu$ m.

be seen that, despite the disappearance of microtubules, these cells were still highly immunoreactive with anti-MAP 2. In contrast to the patterns obtained in control cells, the anti-MAP 2 antibody was now found to stain intermediate filament cables. These cables were found to be immunoreactive with an anti-vimentin antibody, further confirming their identification as intermediate filament-containing structures. Antibodies to the subunit proteins of neurofilaments did not stain these cells. These data are consistent with the identification of the cells as neuronal precursors rather than mature neurons and imply that MAP 2 appears in neurons before the neurofilament proteins.

These results indicated that MAP 2 can bind to intermediate filaments in cells and suggested that one of the functions of this particular protein is to cross-link microtubules to intermediate filaments in the cell. A considerable body of evidence indicates that the two filament systems—microtubules and intermediate filaments—do, indeed, interact in cells (1, 16, 17, 22, 28, 46). Our findings suggest that MAP 2 plays a role in this interaction.

In similar experiments conducted with monoclonal anti-MAP 1A, no evidence for an interaction of this MAP with intermediate filament cables was obtained (7). In fact, in

vinblastine-treated brain cells of the type shown in Fig. 5 that were double-labeled with anti-MAP 2 and anti-MAP 1A, only MAP 2 was found associated with intermediate filament cables (Bloom, G. S., F. C. Luca, and R. B. Vallee, unpublished results). MAP 1A immunoreactivity was spread throughout the cytoplasm. These results indicate that the interaction of MAP 2 with intermediate filaments is specific to only this protein. This contrasts with the results of *in vitro* reconstitution experiments conducted by LeTerrier et al. (29), who found that all the high molecular weight brain MAPs became associated with purified neurofilaments at low stoichiometry.

#### FUNCTIONS OF MAP 2 IN NEURONS

Though there may be more to be learned about the biochemical properties of MAP 2, what we have learned so far about its cellular and subcellular distribution permits us to begin to speculate on its role *in vivo*. The two findings described above—that MAP 2 is most prominent in dendrites and that MAP 2 immunoreactivity is associated with intermediate filament cables—seem to us to provide insight into the fundamental properties of the protein and should provide the basis for further biochemical work. However, these findings also present a paradox. Although intermediate filaments are present in dendrites, they are known to be considerably more abundant in axons (see, for example, reference 41). If the only role for MAP 2 in the neuron were to cross-link neurofilaments with microtubules, it is difficult to understand why the distribution of MAP 2 would not reflect that of the neurofilaments.

It is possible that MAP 2 simply serves to link those neurofilaments that are present in dendrites to the dendritic microtubules. In axons, where MAP 2 is much less abundant than in dendrites (though perhaps not totally absent, as discussed below), some other protein could perform the same function. Candidates for this role would include the MAP 1 polypeptides (Fig. 1) and the component proteins of the neurofilaments.

An alternative interpretation, and one that we favor, is that MAP 2 serves more than one function in neurons. Here, two possibilities may be entertained. MAP 2 could perform separate functions in the axon and the dendrite; or, it could perform different functions at different stages in the development of the neuron. These options are discussed below.

**DENDRITIC CYTOSKELETON:** The most noteworthy feature of the dendritic cytoskeleton is the abundance of microtubules, all arrayed parallel to the longitudinal axis of the dendritic process (see, for example, reference 41). Neurofilaments are less abundant than microtubules. (Little is known about the distribution of actin filaments in the dendrite). The dendritic microtubules are quite widely separated, but, nonetheless, their high degree of order suggests that they interact in some way. Metal-shadowed preparations of MAP 2-containing microtubules have revealed that the MAP arm may extend as far as 90 nm from the microtubule surface (61). The arm thus appears to be sufficiently long to span the distance between adjacent microtubules in the dendrite, estimated to be  $\sim$ 90 nm center to center (43). In view of these considerations and what we estimate to be the very high concentration of MAP 2 in the dendrite (12, 32), it is tempting to speculate that MAP 2 plays a role in the organization of the dendritic microtubules. While there is little evidence at present in support of a second microtubule-binding site in the

MAP 2 molecule (see Fig. 2) or for a self-association of MAP 2 arms, perhaps with further work such evidence will be obtained. It is also possible that MAP 2 serves as a passive spacer separating microtubules, something that may be difficult to prove using *in vitro* biochemistry.

**CYTOSKELETON OF AXONS AND UNDIFFERENTIATED NEURONS:** Evidence has been obtained indicating that during the differentiation of neurons the composition of the intermediate filaments changes. Undifferentiated neurons were found to contain vimentin filaments, which subsequently disappeared and gave way to neurofilaments (2, 23, 65). Our results indicating an association of MAP 2 with intermediate filaments were obtained with primary cultures of neonatal rat brain cells. The subpopulation of cells that was analyzed in our study had properties more characteristic of undifferentiated than of differentiated neurons (4; see above). For example, the intermediate filament cables in these cells reacted with anti-vimentin, but we did not observe reactivity with anti-neurofilament antibodies. Thus, our results indicate that MAP 2 is involved in cross-linking intermediate filaments of the vimentin type to microtubules during the early stages of neuronal differentiation. In the mature neuron, MAP 2 could play an unrelated role, predominantly in the dendrite, as described in the preceding paragraph.

However, it is also possible that, in the mature neuron, MAP 2 does, indeed, function in cross-linking intermediate filaments of the neurofilament type to microtubules. Our finding of low levels of MAP 2 in bovine white matter (Fig. 4) suggests a role for the protein outside of dendrites, despite the absence of detectable immunoreactivity in axons or glial cells (12, 32). Pappasozomenos and co-workers (39) have recently detected low levels of MAP 2 immunoreactivity in mature peripheral axons, indicating that MAP 2 may, indeed, be present in at least some axons. In rats treated with the neurotoxic agent  $\beta, \beta'$ -iminodipropionitrile, which induces a segregation of neurofilaments and microtubules into distinct domains within the axon, MAP 2 immunoreactivity colocalized with neurofilaments (39). While it is not clear why immunoreactivity was not found associated with microtubules, this result appears to support an association of MAP 2 with neurofilaments as well as vimentin filaments.

Thus, MAP 2 may be present in axons as well as dendrites and may play different roles in the two subcellular compartments.

**FUNCTION OF MAP 2 PHOSPHORYLATION:** How can we explain multiple roles for MAP 2? One possible explanation is that phosphorylation specifies either the functional state of the protein, its subcellular localization, or both. Thus, phosphorylation could specify whether the MAP 2 arm interacted with other microtubules or with intermediate filaments. Alternatively, or in addition, phosphorylation could alter the affinity of MAP 2 for some component of the axonal or dendritic cytoskeleton, resulting in the observed differential distribution of the protein in the neuron.

It is worth noting in this context that MAP 2 may be phosphorylated by more than one kinase. Inorganic phosphate analysis of MAP 2 as isolated and following dephosphorylation or rephosphorylation with the associated cAMP-dependent protein kinase (Fig. 3) revealed that only 13 of approximately 21 phosphates could be accounted for by the cAMP-dependent enzyme (53). We proposed that the remaining phosphates were introduced by an, as yet, unidentified kinase. Recently, Yamauchi and Fujisawa (64) did, in fact,

report that a calmodulin-dependent protein kinase isolated from brain tissue was also capable of phosphorylating MAP 2.

It is also worth recalling that analysis of the distribution of phosphorylation sites on the MAP 2 molecule (Fig. 2) suggested that both its microtubule assembly-promoting activity as well as the interaction of the arm domain with other cytoplasmic structures may be regulated by phosphorylation. Workers in two laboratories have already reported an effect of phosphorylation on microtubule assembly (26, 30), though it is not yet certain whether it was the phosphorylation of MAP 2 or of some minor MAP species that was responsible for the observed effect. Nonetheless, it seems reasonable to expect that phosphorylation, perhaps catalyzed by multiple kinases and occurring at multiple sites, will ultimately prove to play a role in regulating several aspects of the behavior of MAP 2.

**INTERACTION OF MICROTUBULES WITH ACTIN FILAMENTS:** Our speculations regarding the function of MAP 2 *in vivo* are based on information obtained by analysis of the cellular and subcellular distribution of MAP 2 and from a structural analysis of the protein. Pollard and co-workers (18), as well as Sattilaro et al. (45) and Nishida et al. (37), have obtained evidence that MAP 2 can interact with actin filaments *in vitro*. This suggests yet a further role for MAP 2, that of mediating the interaction of microtubules and actin filaments. No evidence for such a role for MAP 2 has as yet been obtained from examination of cells. In addition, the restricted cellular and subcellular distribution of MAP 2 suggests that this protein may be an unlikely candidate for what would be expected to be a rather general MAP function. It seems appropriate, in view of the multiplicity of high molecular weight MAPs (Fig. 1) and the low affinity of the MAP 2-actin interaction (18) to reserve judgement on this question until more is known about the properties of the other MAPs.

### *Microtubule-Associated Proteins of the Mitotic Spindle*

Most work in the microtubule field has been with microtubules and MAPs isolated from brain tissue. As described above, at least one major MAP in brain—MAP 2—is highly restricted in its distribution. MAP 1A is more widespread and is found generally in interphase microtubules and mitotic spindle microtubules in a wide variety of cells. Thus, some general information regarding the properties of microtubules has come from work using brain as an experimental system. However, particularly in view of the restricted distribution of MAP 2, it seemed to us desirable to obtain a preparation of microtubules that are primarily involved in mitosis if we are ultimately to understand how the spindle functions.

To this end we have used taxol (55) to prepare microtubules from eggs of the sea urchins *Lytechinus variegatus* and *Strongylocentrotus purpuratus* (59). The microtubules contained numerous apparent MAPs, with an overall electrophoretic pattern quite different from the MAPs of brain tissue. To demonstrate directly that the proteins we had isolated were associated with microtubules in the cell, we produced monoclonal antibodies to a whole MAPs fraction as well as to individual MAP polypeptides. To date we have isolated hybridoma lines secreting antibodies to proteins of  $M_r$  235,000, 205,000, 150,000, 77,000, and 37,000, all of which stain the mitotic spindle of dividing sea urchin eggs (Fig. 6) (5, 59).

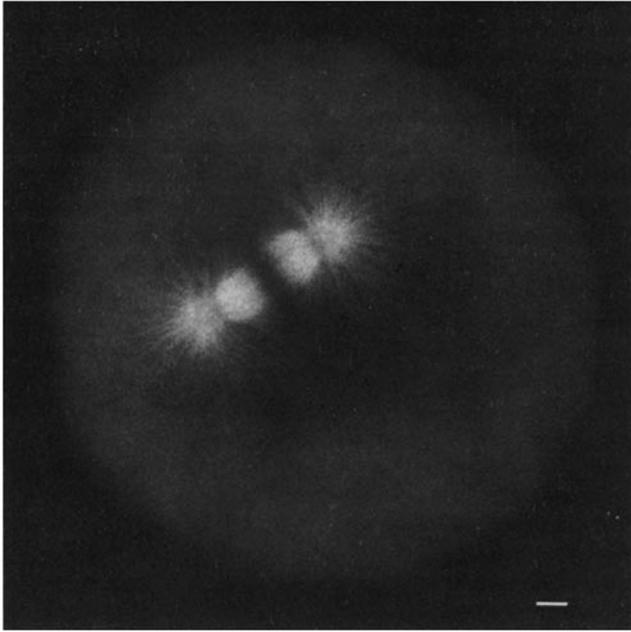


FIGURE 6 Immunofluorescence micrograph of a dividing sea urchin egg (*L. variegatus*) stained with a monoclonal antibody to a 37,000-dalton egg MAP. From Vallee and Bloom (59). Bar, 10  $\mu$ m.

The characterization of this new microtubule system, which is suitable both for biochemical and cellular analysis, is just getting under way. However, the results obtained to date have already revealed that the mitotic spindle is quite complex with regard to its protein composition. In addition, they have revealed that, as has been known for tubulin, MAPs are stockpiled in the unfertilized egg. We hope eventually to generate a complete catalogue of spindle MAPs and determine the function of these proteins in microtubule-assembly regulation and spindle function.

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