Interactions between Neurofilaments and Microtubule-associated Proteins: A Possible Mechanism for Intraorganellar Bridging

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ABSTRACT Mammalian neurofilaments prepared from brain and spinal cord by either of two methods partially inhibit the in vitro assembly of microtubules. This inhibition is shown to be due to the association of a complex of high molecular weight microtubule-associated proteins (MAP_1 and MAP_2) and tubulin with the neurofilament. Further analysis of the association reveals a saturable binding of purified brain MAPs to purified neurofilaments with a K_d of 10^{-7} M. Purified astroglial filaments neither inhibit microtubule assembly nor show significant binding of MAPs. It is proposed that the MAPs might function as one element in a network of intraorganellar links in the cytoplasm.

A number of studies have suggested the existence of interactions between microtubules and neurofilaments. Morphological studies have shown cross-bridges between the two structures (3, 20, 28, 32), and axonal flow studies have shown that tubulin and neurofilament proteins are associated in the slowest component of axonal flow (10). In vitro, biochemical interactions between microtubules and neurofilaments have been suggested by Berkowitz et al. (2) on the evidence of the copurification of the neurofilament triplet protein during the polymerization-depolymerization preparation of brain microtubules. Viscometric and sedimentation analyses have also shown ATP-mediated interactions between the two structures (21), and we have recently found that a microtubule-associated protein (MAP) kinase was able to preferentially phosphorylate one of the neurofilament subunits in a cAMP-dependent manner (16).

The microtubule has been found to contain lateral projections in vitro, which consist of the high molecular weight MAPs. These MAPs bind periodically to the microtubule wall (1, 12). The neurofilament on the other hand appears to consist of a filamentous core which is composed of the 70,000-dalton subunit, with two associated protein (150,000- and 200,000-dalton neurofilament subunits) located more peripherally (8, 14). Recent immunological evidence has suggested that the 200,000-dalton subunit may wind helically around this core (24, 31).

To understand the interactions between neurofilaments and microtubules in vitro, one should therefore study the interactions between each of the microtubule proteins and the neurofilament as well as the neurofilament triplet subunits. In this paper, we present evidence for interactions between purified neurofilaments and high molecular weight MAPs.

MATERIALS AND METHODS

Preparation of Microtubules

Microtubules were purified from bovine brain by the polymerization-depolymerization method of Shelanski et al. (25) as modified by Karr et al. (11). The brains were homogenized in 0.32 M sucrose, 1 mM EGTA, pH 7.0, in a Sorvall Omnimixer (Sorvall-DuPont Co., Newtown, CT) (three 30-s pulses at setting 1.5). The homogenate was centrifuged for 1 h at 105,000 g at 4°C and the supernatant was used to purify microtubules as described by Karr et al. (11) through two cycles of polymerization. The final pellets were disassembled at 4°C and stored at −80°C in reassembly buffer (RB), which consists of 0.1 M MES (2[N-Morpholino] ethane sulfonic acid), 1 mM EGTA, and 1 mM $MgCl_2$, containing 8 M glycerol.

Preparation of Neurofilaments

Bovine brain neurofilaments were purified from the same starting tissue used in the purification of the microtubule proteins. The first pellet obtained after centrifugation of the homogenate was vigorously rehomogenized in an equal volume of 0.32 M sucrose, 1 mM EGTA, pH 7.0, in the Sorvall Omnimixer (three 30-s pulses at setting 9). After centrifugation for 30 minutes at 48,000 g, the supernatant containing the neurofilaments was immediately brought to 0.8 M KCl by the addition of solid salt. The homogenization and centrifugation steps were repeated once on the pellet, and the second supernatant made 0.8 M in KCl was added to the first extract and frozen (−20°C). This step was found to help remove some of the tubulin which contaminated the neurofilaments. The final purification of the neurofilaments was achieved by layering the extracts on a discontinuous sucrose gradient (0.85, 1, and 2 M) made in RB containing 4 M glycerol and 0.8 M KCl. After a 2-h centrifugation (SW 41, 40,000 rpm; Beckman Instruments, Palo Alto, CA), neurofilaments were recovered from the 0.85–1 M

interface and 1–2 M interface. The purified filament suspension was diluted 10-fold with RB containing 4 M glycerol and 0.8 M KCl, and the filaments were harvested by centrifugation at 60,000 rpm for 1 h (Ti 60 rotor). The neurofilament pellets were resuspended by gentle sonication in RB containing 2 M glycerol.

Neurofilaments from rabbit intradural nerve roots were obtained by the method of Liem et al. (13). Nerve roots were carefully dissected, homogenized in 0.85 M sucrose in 10 mM phosphate buffer containing 0.1 M NaCl and 1 mM EGTA, pH 6.8. The homogenate was centrifuged at 10,000 rpm for 10 minutes in a SW 27 rotor. The myelinated axons, which floated to the top of the sucrose layer, were rehomogenized in the presence of 1% Triton X-100 to remove the myelin. The neurofilaments were collected by centrifugation at 270,000 g for 30 min and further purified on a discontinuous sucrose gradient as described (13). The purified neurofilaments were recovered from the gradient, centrifuged for 1 h at 60,000 rpm, and resuspended by sonication in RB containing 2 M glycerol.

Neurofilaments from bovine spinal cord were prepared by the method of Delacourte et al. (4, 5). Spinal cords were homogenized in RB (1:1 wt/vol) in Sorvall Omnimixer 3 × 30 in, at setting 9. The homogenate was centrifuged at 40,000 rpm for 30 min at 4°C in a Ti 60 rotor. The supernate was brought to 4 M glycerol and kept at 0°C for 30 min before centrifugation. The neurofilament pellet, obtained after centrifugation at 40,000 rpm for 1 h in a Ti 60 rotor, was resuspended in RB containing 1 M NaCl. This material was further purified by centrifugation through 1 M sucrose in MES buffer, containing 0.1 M NaCl, for 2 h at 50,000 rpm in a Ti 60 rotor to remove membrane contaminants. The final pellet of purified neurofilaments was resuspended in RB containing 2 M glycerol.

Purification of Glial Filaments

Glial filaments were purified from bovine brain white matter according to the method of Liem (15). Crude intermediate filaments were obtained by the axonal flotation method, followed by removal of the myelin and membranes by treating with Triton X-100. The filaments were dissolved in a 10 mM phosphate buffer, pH 7.4, containing mercaptoethanol, made up in 8 M urea. The solubilized filaments were chromatographed on hydroxylapatite. Glial filament proteins eluted in the void volume, whereas the other proteins stuck to the column and were removed with higher ionic strength buffers (15). Glial filaments were reassembled from this mixture by dialysis against RB and collected by centrifugation at 40,000 rpm for 60 min in a Ti 60 rotor. The filaments obtained in this manner were resuspended by sonication in RB containing 2 M glycerol.

Purification and Phosphorylation of High Molecular Weight MAPs

Microtubules purified by two cycles of polymerization-depolymerization were separated into tubulin and MAP fractions by phosphocellulose chromatography as described by Weingarten et al. (29). The MAP fractions, which eluted from the column with 0.3 M NaCl, were pooled, concentrated by ammonium sulfate precipitation (60% of saturation), and resolubilized in RB containing 0.8 M KCl. The concentrated protein sample was loaded on an AcA 34 Ultrogel column equilibrated with RB containing 0.8 M KCl. The separation of high molecular weight MAPs (HMWs) and the smaller molecules was monitored by SDS PAGE of the column fractions. The HMW-containing fractions were pooled and concentrated by precipitation with ammonium sulfate, redissolved in RB and desalted on a Sephadex G-25 column equilibrated with RB. The purified HMWs were stored at −80°C in RB containing 2 M glycerol.

For the phosphorylation procedure, 1 ml of purified HMW protein (0.3–0.5 mg/ml) was incubated for 30 min at 37°C in the presence of 2×10^{-5} M γ-^{32}P-ATP (2.5 μc/nmol) and 5×10^{-6} M cAMP. At the end of the incubation, the sample was loaded on a Sephadex G-25 column equilibrated with RB containing 2 M glycerol to remove the unbound radioactive ATP. A sample of the labeled protein was precipitated on a square of Whatman 3 MM paper, soaked in 10% TCA, and the TCA-precipitable protein was determined as previously reported (16). The purified ^{32}P labeled HMW proteins were either used immediately for the binding experiments or stored at −80°C.

Binding of ^{32}P-HMW Protein to the Neurofilaments

The purified labeled MAPs (0.2–0.3 mg/ml, 5×10^6 cpm/mg) were incubated with rabbit intradural root neurofilaments at 37°C. To measure the HMW bound to the neurofilaments, the samples were loaded on a sucrose layer and centrifuged for 1 h at 60,000 rpm in a SW 60 swinging bucket rotor at 4°C. The supernatants were carefully separated from the pellets, which were dissolved either in 0.1 N NaOH for counting or in gel sample buffer. The radioactivity of both fractions was determined by Cerenkov counting (40% efficiency). The amount of HMW bound/μg neurofilament was determined from the ratio of radioactive ^{32}P in the

supernatant to that in the pellet. The background were determined by incubating the ^{32}P-labeled MAPs in the absence of neurofilaments. Control experiments measured the binding of ^{125}I-labeled alkaline phosphatase to neurofilaments using alkaline phosphatase, which was iodinated by the Chloramine T procedure as described elsewhere (13).

Gel Electrophoresis and Autoradiography

6–15% gradient and 7.5% polyacrylamide gels containing 0.1% SDS were used for the electrophoresis experiments (12). Gels were fixed with 50% methanol and 10% acetic acid, stained with Coomassie Blue, and destained with 10% acetic acid. After destaining, the gels were dried and exposed to Kodak XR3 film. Protein measurements were made according to Lowry et al. (17).

RESULTS

Polymerization of Microtubules in the Presence of Purified Neurofilaments

The addition of increasing quantities of bovine brain neurofilaments to a mixture of brain microtubule proteins prepared by two cycles of assembly and disassembly results in a partial inhibition of microtubule assembly as measured by turbidimetry at 340 nm (7). Decreases both in the initial rate of assembly and in the extent of polymerization are observed (Fig. 1). This inhibitory effect on microtubule assembly is dependent on the concentration of neurofilaments added to the mixture. For control experiments, we studied the effect of glial filaments on microtubule assembly in the same range of concentrations and found them to have no effect (Fig. 1, *inset*).

When one cycle of microtubule assembly in the presence of neurofilaments is followed by disassembly at 4°C and centrifugation at 100,000 g for 30 min in the cold to remove filaments, a subsequent cycle of microtubule assembly shows inhibition proportional to the filament concentration in the initial polymerization mixture (Fig. 2). This inhibition is more marked in the initial velocity than in the extent of the reaction. These observations suggest an interference of neurofilament protein in microtubule polymerization, which could be due either to a loss of the microtubule accessory proteins (MAPs) or to a loss of nucleation centers. Electrophoretic examination of the assembly mixtures in this experiment revealed that (*a*) there is
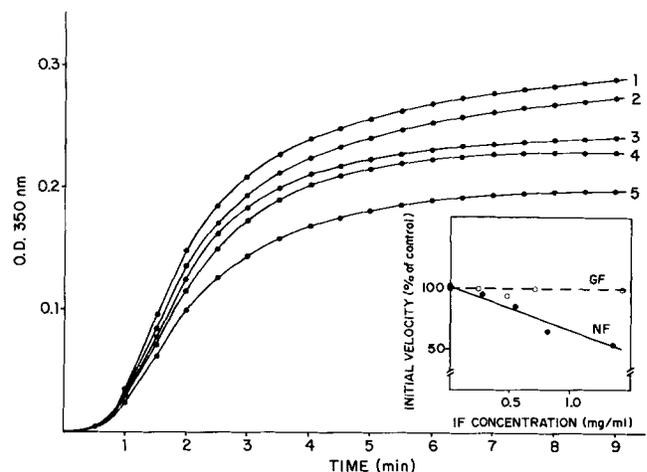

FIGURE 1 Polymerization kinetics of microtubules in the presence of bovine brain neurofilaments: *1*: control. *2*: 0.05 mg/ml. *3*: 0.26 mg/ml. *4*: 0.5 mg/ml. *5*: 0.77 mg/ml neurofilaments. Tubulin concentration 1.82 mg/ml. *Inset*: Inhibition of the initial velocity of microtubule polymerization by spinal cord neurofilaments (*NF*) and reassembled glial filaments (*GF*). Microtubule concentration: 1 mg/ml.
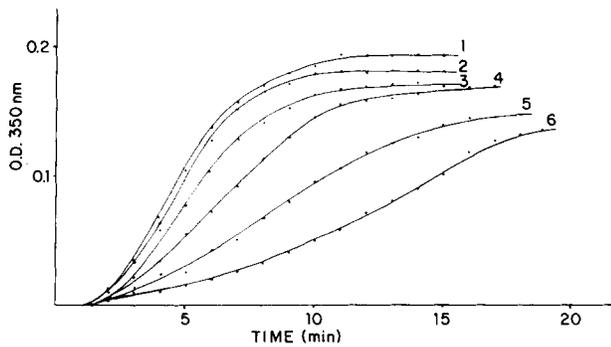
FIGURE 2 Polymerization kinetics of supernate obtained after two-cycled microtubule proteins are incubated with neurofilaments, followed by removal of the filaments by centrifugation. Neurofilament concentrations: 1: 0 mg/ml. 2: 0.14 mg/ml. 3: 0.28 mg/ml. 4: 0.55 mg/ml. 5: 0.83 mg/ml. 6: 1.1 mg/ml. Microtubule concentration, 1.5 mg/ml.

no major change in the tubulin concentrations as a result of removing the neurofilaments, although the protein concentration of the supernatant was slightly decreased (2–5%) for the highest neurofilament concentration in the previous cycle, (b) there is no neurofilament triplet remaining in the supernates, and that (c) there is a significant decrease in high molecular weight MAPs which appears to be proportional to the amount of neurofilament protein in the original cycle of assembly (Fig. 3 a). These results suggest binding of HMWs to the neurofilaments, which were removed by centrifugation, lowering the MAP concentration in the microtubule mixture, thereby lowering the initial velocity of microtubule polymerization, with a less significant effect on the extent of the reaction. However, when the neurofilaments removed from the assembly mixture were analyzed by SDS PAGE, a small amount of tubulin was found together with the MAPs in these fractions (Fig. 3 b). These findings could therefore be interpreted as an association between the neurofilaments and a MAP-tubulin complex, which is involved in the initiation of tubulin polymerization.

Binding of MAPs to Neurofilaments

To study the binding of the MAPs to the neurofilaments, HMWs were purified and phosphorylated. These preparations contain MAP$_1$ and MAP$_2$, the latter being the only labeled molecule (Fig. 4 b, far right).

In the binding assays, purified ^{32}P-labeled HMWs were incubated with rabbit nerve root or bovine spinal cord neurofilaments at 37°C for various times at different concentrations. After incubation, the insoluble neurofilaments were separated by centrifugation through a sucrose layer, and the radioactivity in both the pellet and supernatants was measured. The radioactivity in the pellet was exclusively from the neurofilament-bound MAPs, as revealed by an autoradiogram of the gel electropherogram of the samples (Fig. 4 b). The Coomassie-Blue-stained pattern of these samples showed that both MAP$_1$ and MAP$_2$ were bound to the neurofilaments (Fig. 4 a). As shown in Fig. 5 a, rapid binding of the HMW proteins to the filaments is observed. At 0°C, the binding was <10% of the value at 37°C. When increasing concentrations of HMW proteins were incubated with a fixed concentration of neurofilaments, binding is seen to increase to a plateau value (Fig. 5 a). This maximum binding approached 0.015 mg of HMW protein/mg of neurofilament triplet protein. Control experiments under the same conditions in which HMW proteins were

replaced by ^{125}I-labeled alkaline phosphatase showed no binding of radioactive label to the neurofilaments. When glial filaments were incubated with the ^{32}P-MAPs, only a low level of binding was observed, with no apparent saturation (Fig. 5 b). When the amount of bound MAPs vs. initial MAP concentration was replotted in a double reciprocal plot (Fig. 6), a dissociation constant for binding of $\sim10^{-7}$ M is observed, assuming a molecular weight of 300,000 daltons for HMW proteins. The binding capacity obtained from this plot shows 0.015 mg HMW/mg neurofilament.

DISCUSSION

The partial inhibition of in vitro microtubule assembly by intact neurofilaments raises the possibility of direct interactions
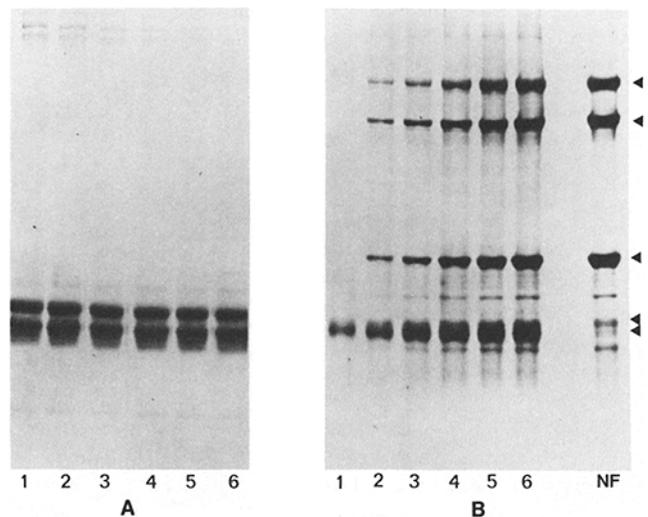

FIGURE 3 (A) Gel of polymerized microtubules described in Fig. 2. (B) gel of the proteins pelleted prior to assembly shown in Fig. 2. Neurofilament concentrations in the different lanes as in Fig. 2. NF is neurofilament control. Arrowheads indicate (from top): NF200, NF150, NF70, and tubulin dimer.
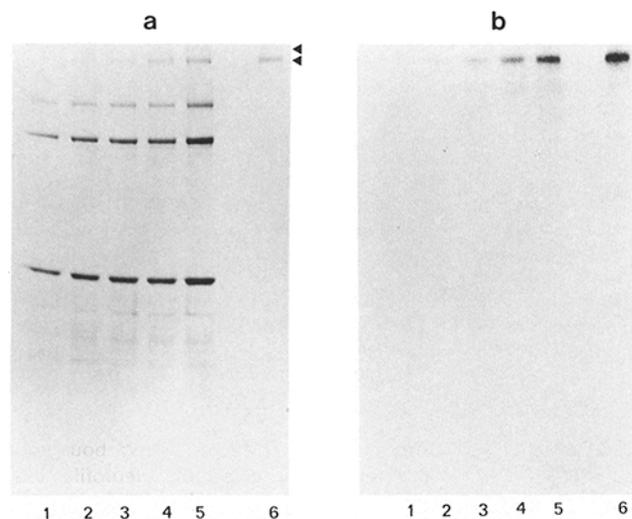

FIGURE 4 (a) Gel and (b) autoradiogram of phosphorylated HMW proteins bound to the neurofilaments. Lanes 1–5: increasing MAP concentrations from 0.019 mg/ml to 0.115 mg/ml; NF concentration: 0.108 mg/ml. Lane 6: ^{32}P-MAPs alone (0.4 µg). Arrowheads indicate the positions of MAP$_1$ and MAP$_2$.
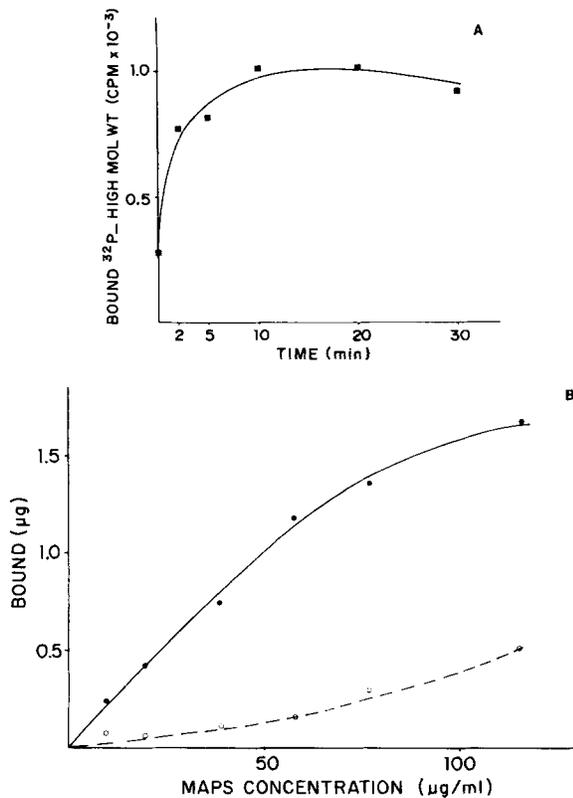
FIGURE 5 (a) Kinetics of binding of ^{32}P-HMW to nerve root neurofilaments. Protein concentrations are HMW: 0.02 mg/ml and NF 0.054 mg/ml. The incubation was started by changing the temperature to 37°C and stopped by loading the samples on a sucrose layer, followed by immediate centrifugation at 4°C. (b) Binding of increasing concentration of ^{32}P-HMW (solid line) to spinal cord neurofilaments (0.108 mg/ml). The samples were incubated for 30 min at 37°C before centrifugation. A control experiment with reassembled glial filaments (dashed line) was performed under the same conditions.
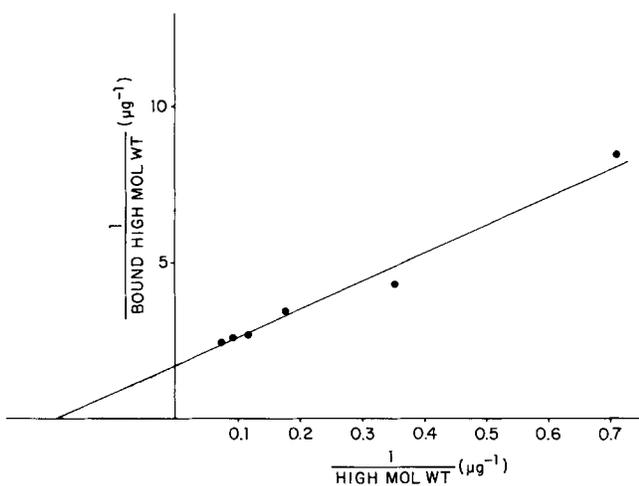

FIGURE 6 Double reciprocal plot of HMW vs. HMW bound to neurofilaments from rabbit intradural nerve roots. Neurofilament concentration: 0.054 mg/ml.

between the two classes of organelles. Such interactions have been suggested by the copurification of tubulin and neurofilaments under certain conditions (2), by electron micrographs showing links between fibrillar organelles, by low shear viscom-

etry demonstrating ATP-mediated interactions between microtubules and neurofilaments (21), and by the demonstration of MAP$_2$-mediated phosphorylation of the 150,000-dalton component of the neurofilament (16). Electron micrographs support bridging between the organelles (26), perhaps mediated by the MAP-containing arms on the microtubules, or the neurofilament accessory proteins, which may be represented by the 150,000- and 200,000-dalton components of the neurofilament triplet (14, 31). The inhibition seems to involve the association to the neurofilaments of a small proportion of the microtubule proteins present in the mixtures, including the HMW MAPs as well as a small amount of tubulin (Fig. 4). A possible mechanism raised by these observations is that a complex of MAPs-tubulin, essential for the microtubule polymerization process, is bound to the neurofilaments and hence removed from the assembly mixture. This complex may be similar to the one described for the initial part of the microtubule polymerization reaction (27). This hypothesis is further supported by the lack of a significant change in the tubulin concentration of assembly mixtures after removal of neurofilaments, which suggests that the mechanism of the continued inhibition of assembly is an increased critical concentration. This inhibition involves MAPs as demonstrated by the decrease in amount of HMWs in the mixtures as a function of filament concentration in the initial assembly cycle (Fig. 4).

The results of the binding experiments between purified phosphorylated HMW proteins and neurofilaments showed that binding occurred with high affinity ($K_d \cong 10^{-7}$–5×10^{-7} M) and that the binding was saturable. This binding constant is similar to the half-concentration of MAP$_2$ required to achieve the half-maximal polymerization of tubulin (22), although this value does not describe the affinity constant of MAP$_2$ for tubulin, which may be higher during the formation of nucleation centers (27). The specificity of the binding of HMWs to the neurofilaments is confirmed by the lack of binding of another basic protein (alkaline phosphatase) under the same conditions and by the very poor, nonsaturable association of the MAPs to glial filaments.

These results are consistent with our previous work which described the phosphorylation of the 150,000-dalton neurofilament component by the cAMP-dependent protein kinase associated with MAP$_2$, since both sets of results suggest interactions between the neurofilament triplet and the HMWs.

The recent immunological studies of Willard and Simon (31) and of Sharp et al. (24) and the reassembly studies of Liem and Hutchison (14) and of Geisler and Weber (8) have suggested that the neurofilament is composed of a core of 70,000-dalton subunit and that the other two polypeptides serve as accessory proteins. The binding data presented here, in combination with the phosphorylation study reported previously, suggest that the interactions between neurofilaments and microtubules may be represented as arm-to-arm binding rather than arm-to-core. Further experiments using the isolated individual subunits of the neurofilament and phosphorylated HMW proteins should elucidate this mechanism. The interactions between microtubules and neurofilaments could be a cell type-specific interaction. Recently, HMW proteins similar to MAPs have been described associated with intermediate filaments from different cell lines (19). It would be interesting to see whether these accessory proteins also form the basis of the interactions between the cytoskeletal elements in these cells, or whether the neurofilament-microtubule interactions found here are specific to the neuron.

Our findings are in accord with morphological observations showing extensive "bridging" between microtubules, intermediate filaments, other cytoplasmic organelles, and plasmalemmal or subplasmalemmal structures (3, 20, 28, 32). They reinforce existing biochemical (16, 21) and biophysical data on MAP mediation of mictotubule-neurofilament interactions. Similar roles for MAPs in mediating interactions of microtubule and actin (9, 18, 23) and between actin filaments have also been suggested by biochemical approaches. These data point toward the MAPs as one member of the class of intraorganellar bridges or the microtrabecular lattice (6). Such a role is also suggested by the recent immunological localization of the intermediate filament-associated HMW protein "plectin" (30).

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