Microtubule-associated Proteins Bind Specifically to the 70-kDa Neurofilament Protein*

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Morphological and biochemical evidence have suggested that the components of the neuronal cytoskeleton, microtubules and neurofilaments (NF), interact with each other. Microtubule-associated proteins (MAPs) are plausible candidates for mediating some of these interactions and have been shown to bind to neurofilaments, as well as induce the formation of a viscous complex between neurofilaments and microtubules. By binding 32P-labeled MAPs to neurofilament proteins, which were transferred electrophoretically to nitrocellulose, we determined that, of the three neurofilament subunits, only the core NF70 subunit bound MAPs. The binding to electrophoretically transferred NF70 was specific, saturable, and reversible. Binding parameters were estimated by binding 32P-labeled MAPs to purified NF70 immobilized on nitrocellulose. Approximately 1 mol of MAPs bound per 45 ± 15 mol of NF70 with an approximate Keq = 2.0 ± 0.9 × 10^2 M (n = 8). Reassembled filaments in suspension were used to confirm the specific binding. Tubulin and NF70 apparently bind to different sites on MAPs.

Neurofilaments (NF) and microtubules are major components of the neuronal cytoskeleton. The neurofilaments are 8–10 nm in diameter and consist of three polypeptides of 70 kDa (NF70), 150 kDa (NF150), and 200 kDa (NF200) (1, 2). Biochemical assembly studies have shown that NF70 can assemble by itself into filaments, whereas the other two subunits coassemble with NF70 (3, 4). These data along with immunoelectronmicroscopic studies (5–7) have led to the assignment of NF70 as the “core” of the filament and NF200 and NF150 as peripheral proteins. The biochemical properties of NF200 and NF150 and their peripheral location along the filament make them prime candidates for interelemental linkers in the cytoskeleton. Recent data suggest a role for NF200 as a filament-filament linker (6, 8).

Microtubules are composed of tubulin, a heterodimer of α- and β-tubulin with 53 and 55 kDa, respectively (9, 10). Several microtubule-associated proteins (MAPs), high molecular mass microtubule-associated proteins with 300 to 300 kDa (MAP1 and MAP2) and “r” proteins with 50–70 kDa, are tightly associated with microtubules and promote the assembly of tubulin into microtubules (11–13). Side arm projections from the tubular structures can be seen in microtubules formed in the presence of MAPs (14) or by the addition of MAPs to preformed smooth-walled microtubules (15).

For the cytoskeleton to perform the complex roles in which it is implicated, carefully regulated interactions among the various elements are probably necessary. Morphological analysis of the cellular cytoskeleton (16) clearly shows that the various elements are not independent, but rather integrated. Links between microtubules and synaptic vesicles (17), microtubules and mitochondria (18–20), microtubules and the membrane (21, 22), tubules and filaments (23, 24), and adjacent tubules (25) have been observed.

Axonal transport studies (26), viscometric studies of a neurofilament-microtubule complex (27, 28) as well as direct interaction studies between neurofilaments and MAPs (29) provide evidence for the existence of interactions between these two cytoskeletal elements. Microtubule-microfilament and microtubule-neurofilament interactions have recently been shown to be MAP-mediated (23, 24, 29). MAP2 has been found to resemble α-spectrin immunologically (30) indicating that the cytoskeleton and the membrane skeleton may be composed of a related “family” of proteins.

We have undertaken to identify the component(s) of the neurofilament that have the binding site for MAPs and which might be involved in the in vivo interactions between neurofilaments and microtubules.

**MATERIALS AND METHODS**

Intermediate Filaments and MAPs

Neurofilament and glial filament proteins (GFPs) were obtained from bovine spinal cord by the method of Liem and Hutchison (4). MAPs were prepared from bovine gray matter. 3 × cycled microtubules (32) were stabilized with 10 μM taxol and MAPs eluted with 0.35 M NaCl (33). Phosphorylation of MAPs was performed as described by Leteirier et al. (29). To minimize the possibility of conformational changes as a result of phosphorylation, 1 mg/ml of MAPs were incubated with 20 μM [32P]adenosine 5′-triphosphate (specific activity ~ 10 Ci/mmol, New England Nuclear) and 5 μM cAMP in 0.1 M MES buffer, pH 6.8, containing 1 mM EGTA and 0.5 mM MgCl2 (Buffer A) for 30 min at 37°C. Complete phosphorylation of MAPs can be achieved at 1 mM ATP (34, 35). The concentration of ATP used for the phosphorylation of MAPs in these experiments was therefore 1/50 that for maximum phosphorylation and less likely to cause conformational changes in the molecules. The labeled proteins were separated from the free ATP by chromatography on a 10-ml Sephadex G-25 column. Aliquots of fractions were counted and the fraction of trichloroacetic acid-precipitable counts was deter-
mined. Only the fractions with >95% of 32P incorporated in the trichloroacetic acid precipitates were used. The Coomassie Blue stained gel and autoradiogram of phosphorylated MAPs is shown in Fig. 1, lanes a and b, respectively. Purified NF70 (4) was iodinated by the chloramine-T method (31). Fig. 1, lane c shows an autoradiogram of the iodinated NF70. No degradation was detected.

**Binding of MAPs to Neurofilaments**

**Binding to Transblotted Proteins—Central nervous system filament preparation** (36), purified NF70, tubulin (3), gial filament protein (4), vimentin (purified from bovine lens by the method of Geisler and Weber (37), bovine epidermal keratins (kindly supplied to us by Dr. T. T. Sun, New York University School of Medicine), actomyosin, bovine serum albumin, and protein A (Sigma) were separated by SDS-PAGE. Individual lanes were cut out and transferred to nitrocellulose in a Bio-Rad transblot apparatus (38) for 1 h in 0.0025 M Tris, 0.0192 M NaCl (Buffer B), and 2.5% Triton X-100 to remove bound SDS and saturate the nonspecific binding sites on the nitrocellulose. Binding was carried out by overlaying the nitrocellulose strips with either Buffer A or 0.005 M phosphate buffer, pH 7.2, 0.10 M NaCl (PBS) containing 1-2 μg/ml of 32P-MAPs (specific activity = 50,000-100,000 cpm/μg of protein) for 45 min. These conditions were chosen, since they favor assembly of microtubules and neurofilaments and resemble physiological conditions in their pH and ionic strength. Experiments were also conducted to determine both the pH and ionic strength dependence, and we found that at low ionic strength the binding was significant, but independent of pH. At physiological salt concentrations, the binding varied with pH. The binding was strongest at pH 6.2 and very weak at pH 8.2. At pH 7.2, we obtained significant binding and low background. Following the incubation, the samples were rinsed extensively with binding buffer blotted dry and exposed to Kodak X-Omat AR5 film for autoradiography.

**Binding to Proteins on Nitrocellulose Circles—4-5 μg of neurofilaments were incubated on 5-mm diameter nitrocellulose circles in a 96-well micro test tissue culture plate in 25 μl of 0.0025 M Tris, 0.001 M EDTA, pH 8.2 (Buffer B), for 2 h. To saturate nonspecific binding sites, 5% bovine serum albumin containing Buffer B was added. Binding was carried out as with the transblots. 32P-MAPs of lower specific activity (15,000-30,000 cpm/μg of protein) were used to achieve lower background. A similar experiment was done with MAPs immobilized on nitrocellulose and 12161-NF in suspension, except that the MAPs were immobilized in Buffer A and binding was done in PBS.

**Binding to Reconstituted Filaments in Suspension—** Purified NF70, NF70 + NF150 or GFP in 8 M urea were reassembled into filaments by dialysis against PBS and spun in a Beckman L8-80 Ultracentrifuge Ti 70 rotor at 40,000 rpm for 60 min. The pellets were resuspended in Buffer A and aliquots were incubated with varying concentrations of 32P-MAPs at room temperature. Following the incubation, samples were spun for 5 min in an Eppendorf microfuge (12,000 × g). Pellets were rinsed 3 times with Buffer A and dissolved in 8 M deionized urea, boiled in 0.065 M Tris, 10% glycerol, 1% mercaptoethanol, 1% SDS. Aliquots were removed and counted by scintillation counting and the remainder of the sample was subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried, and exposed.

**RESULTS**

**Binding of 32P-MAPs to Proteins Transblotted to Nitrocellulose Membranes—** To identify which component of the neurofilament bound to microtubule-associated proteins, we separated a preparation of central nervous system intermediate filament proteins, which contained NF70, NF150, NF200, and GFP, by SDS-PAGE. The proteins were transferred electrophoretically to nitrocellulose membranes and the membranes were incubated with 32P-MAPs. The protein composition of the transferrered proteins is shown in Fig. 2, lane a. An autoradiogram of a parallel sample, which was incubated with 32P-MAPs is shown in lane b. This autoradiogram, therefore, shows the proteins which bound 32P-MAPs. There was no binding to the higher molecular weight neurofilament proteins NF150 and NF200. Strong binding was detected at 70 kDa and weaker binding at 50 kDa, probably representing binding to NF70 and GFP, respectively. However, binding to tubulin present in trace amounts in the preparation could not be totally ruled out from this experiment. We further confirmed these observations with purified proteins, including NF70 (lane c), as well as tubulin (lane d). Strong binding to these two proteins was detected, whereas there was no binding to purified NF150 and NF200 (not shown). Two other intermediate filament proteins, glial filament protein (lane e) and vimentin (lane f), show binding of 32P-MAPs to a lesser extent than to NF70. Binding of MAPs to keratins (lane g) was comparable to NF70. Insignificant amounts of MAPs bound to myosin (lane h) and none to actin (lane i), bovine serum albumin (lane i), or protein A (lane j). In all experiments, the amounts of protein loaded on the gels and the length of exposure of the autoradiograms were comparable.

The binding of MAPs to NF70 transferred to nitrocellulose after SDS-PAGE is dependent on the amount of protein loaded. Fig. 3a shows the autoradiogram of 32P-MAPs bound to increasing amounts of NF. Parallel samples were stained with Amido Black, (Fig. 3b), showing the amount of NF present in each sample. 2 μg of NF protein was sufficient to visualize the binding. In subsequent experiments, we routinely used 5 μg of protein. Equilibrium was reached in 30 min. The binding could be visualized only if the transferred proteins were incubated prior to binding in buffer containing Triton X-100, indicating that removal of SDS was necessary for this binding. Triton reduced the background as well as enhanced the intensity of the bands.

The binding of 32P-MAPs to NF70, which had been electrophoretically transferred to nitrocellulose, followed the requirements of a biologically specific binding. It approached saturation and was dependent on MAP concentration. In Fig. 4, we plotted the number of counts obtained by excising the NF70 band, solubilizing the MAPs and counting them in a liquid scintillation counter. To account for the increase in background at increasing concentrations of 32P-MAPs, a nitrocellulose band of equal size as the NF70 band was excised.
Neurofilament-MAP Binding

FIG. 2. Specificity of $^{32}$P-MAP binding. Proteins separated by SDS-PAGE (7.5%) were electrophoretically transferred to nitrocellulose and either incubated with $^{32}$P-MAPS or stained with Amido Black. Lane a, Amido Black-stained nitrocellulose of central nervous system intermediate filaments. Lanes b–j, autoradiograms of $^{32}$P-MAPS bound to: lane b, CNS filaments; lane c, NF70; lane d, tubulin; lane e, glial filament protein; lane f, vimentin; lane g, bovine skin keratins; lane h, actomyosin; lane i, bovine serum albumin and lane j, protein A. Arrows point to the positions of molecular weight markers: bovine neurofilament preparation (200,000, 150,000, and 70,000) and GFP (50,000) (2).

From a corresponding lane, solubilized, and counted, and the counts were subtracted from the total. Excess unlabeled MAPs competed out the bound $^{32}$P-MAPS, and the binding shows evidence of saturability.

Binding of $^{32}$P-MAPS to Neurofilament Protein on Nitrocellulose Membranes—To assess the binding quantitatively and eliminate possible artifacts as a result of the electrophoresis in the presence of SDS, we determined conditions for the immobilization of proteins on nitrocellulose membranes in the absence of detergent. Initially, it was necessary to optimize the conditions so that significant and reproducible amounts of NF70 or MAPs will bind to the nitrocellulose. For this purpose, we used radioactively labeled proteins $^{32}$I-NF70 and $^{32}$P-MAPS. The buffer that gave the most satisfactory results for the attachment of the proteins was 0.0025 M Tris, 0.001 M EDTA, pH 8.2, for neurofilaments and Buffer A for MAPs as described under "Materials and Methods." We experimented with various buffers. The low ionic strength basic pH buffer was tried, because it was similar to the Western blot transblotting buffer, and alkaline buffers are frequently used to coat microtiter plate wells in enzyme-linked immunoabsorbent assay (ELISA). Under the conditions that we described, 60 ± 10% of the spotted protein attached to the nitrocellulose circles. The range of the amount of protein at which the binding of NF70 to the nitrocellulose was linear was 2–10 μg of protein/5-mm diameter nitrocellulose circle. Following the attachment of NF70 to the nitrocellulose, the binding studies were performed in Buffer A or PBS, similar to those described for the transblots. As shown in Fig. 5, employing this approach we confirmed dose-dependent saturable reversible binding to NF70, lack of binding to NF150 and NF200, and very low (close to none) binding to glial filament protein. Fig. 5A shows total binding to NF70 (solid squares) and total binding to NF150 and NF200 (open squares). Fig. 5B shows the specific binding to NF70 calculated by subtracting the nonspecific binding from the total binding. In order to determine the nonspecific binding shown in Fig. 5B, the binding was measured in the presence of 25 x unlabeled MAPs. Since it is difficult to prepare large enough quantities of pure MAPs to perform the experiments in the
Fig. 5. Binding of $^{32}$P-MAPs to NF70 immobilized on nitrocellulose circles. A, total binding of $^{32}$P-MAPs to NF70 (○) and to NF150 and NF200 (□). B, specific binding (△ and ●) and nonspecific binding (◊, ○) in the presence of 25-fold excess unlabeled MAPs.

We have presented data which demonstrate that the binding sites for MAPs on the neurofilaments are on NF70, the core of the neurofilament, and not on NF200 or NF150, the peripherally located subunits. These data were obtained by three different procedures. $^{32}$P-labeled MAPs were bound to neurofilaments, which were either transblotted to nitrocellulose after SDS-PAGE, immobilized to nitrocellulose in the absence of SDS, or in suspension as reassembled purified filaments. In all three types of experiments, binding of MAPs to NF70 could be demonstrated.

The use of nitrocellulose membranes to demonstrate these interactions was of great advantage. Binding to individual proteins in a mixture of cytoskeletal proteins was possible after separation by SDS-PAGE and transfer to nitrocellulose, followed by incubation with $^{32}$P-MAPs and autoradiography. Comparisons with parallel Amido Black stained strips allow for the identification of the protein which binds to the $^{32}$P-MAPs. This procedure is similar to the way that one detects an antigen, which an antibody recognizes in a "Western" blot (38). As shown in Fig. 2, we compared various cytoskeletal proteins (neurofilament triplet proteins, glial filament protein, vimentin, keratin, tubulin, actin, and myosin) and binding proteins of wide specificity, i.e. bovine serum albumin and protein A. The results indicate that the binding of $^{32}$P-MAPs to NF70 was a specific interaction and not an artifact caused by nitrocellulose. The binding on nitrocellulose after the electrophoretic transfer was time- and concentration-dependent and reversible, but no quantitative parameters could be obtained from the results, since estimates of the amounts of protein transferred were difficult to obtain.

To confirm specificity and to estimate the binding parameters, we used purified proteins, immobilized to nitrocellulose, that were not exposed to SDS. From these data, the apparent binding ratio is 1 mol of MAPs/45 ± 15 mol of NF70 with an approximate $K_d$ of $2.6 \pm 0.9 \times 10^{-7}$ M ($n = 8$) at pH 6.8. For these estimates, we used 300 kDa as the average molecular mass of MAPs. In the phosphorylation procedure, MAP2 is the predominant protein labeled (Fig. 1); therefore, the results under these conditions (e and f). MAP binding to neurofilaments appears to be saturable (Fig. 7). In the Coomassie stained fractions (Fig. 6, lanes b and d), we can see that in addition to the binding of MAP2, MAP1 binding is also detectable, especially at higher concentrations. MAP2 binding is detectable much earlier in the autoradiogram, as well as in the Coomassie stained gel, but since MAP2 is the most abundant and most phosphorylated species in our preparation, we should detect MAP2 binding more readily. These experiments suggest that MAP1 binding to NF70 also occurred, but it is not clear whether this is through a complex which involves MAP2, or by direct binding of MAP1 to NF70. In addition, since we can detect binding of MAP2 to NF70 in the Coomassie Blue stained gel, and our labeling with $^{32}$P was such that only 1/50 of the maximum MAP2 phosphorylation occurred, these experiments also indicate that the binding is not an artifact due to the additional phosphorylation.

We subsequently attempted to answer the question whether the binding sites on MAPs for neurofilaments and tubules are different or shared. Labeled NF70 was bound to MAPs on nitrocellulose circles in the presence or absence of taxol-stabilized microtubules devoid of MAPs. Results are shown in Table I. Presence of microtubules did not affect binding of NF70 to MAPs. Finally, NF70 did not bind to microtubules in the absence of MAPs. These data indicate that MAPs have different binding sites for the microtubules and for NF70.

**DISCUSSION**

We have presented data which demonstrate that the binding sites for MAPs on the neurofilaments are on NF70, the core of the neurofilament, and not on NF200 or NF150, the peripherally located subunits. These data were obtained by three different procedures. $^{32}$P-labeled MAPs were bound to neurofilaments, which were either transblotted to nitrocellulose after SDS-PAGE, immobilized to nitrocellulose in the absence of SDS, or in suspension as reassembled purified filaments. In all three types of experiments, binding of MAPs to NF70 could be demonstrated.

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Fig. 6. Binding of \(^{32}\)P-MAPs (0.05, 0.1, 0.15, 0.25, 0.5, 1.0, and 2.0 \(\mu\)g/\(\mu\)l) to reconstituted NF70 (a and b), NF70 + NF150 (c and d), and GFP (e and f) in suspension. Concentrations of NF and GFP are 1 \(\mu\)g/\(\mu\)l. a, c, and e, autoradiograms; b, d, and f, Coomassie Blue-stained gels of the pellets. To resolve MAP1 from MAP2, the gel in panels a and b is 6%; others were 7.5%. Arrows point to the position of MAP1, MAP2, NF150, NF70, and GFP (G). Molecular weight markers are the same as in Fig. 2.

Fig. 7. Binding of \(^{33}\)P-MAPs to reassembled neurofilaments and glial filaments. The pellets from the experiments shown in Fig. 7 were dissolved and counted.

in Figs. 4–7 reflect primarily the binding of MAP2 to neurofilaments. However, the MAP fractions that we used also contain MAP1 and \(\tau\) proteins (Fig. 1, lane a), which by themselves could bind or modulate binding. This might be the cause for the variability in the observed binding ratio.

Table I

| Binding of \(^{125}\)I-NF70 to MAPs |
|----------------------------------|
| Triplicate samples of MAPs (20 \(\mu\)g) on nitrocellulose circles were incubated with \(^{125}\)I-NF70 (1 \(\mu\)g). 35 \(\mu\)g of microtubules were added to the respective samples. The amount of \(^{125}\)I-NF bound to nitrocellulose preincubated with bovine serum albumin was considered background. |
| \(^{125}\)I-NF70 bound | % |
| \(^{125}\)I-NF70 + MAPs | 100 |
| \(^{125}\)I-NF70 + MAPs + microtubules | 96 |
| \(^{125}\)I-NF70 + microtubules | 0 |

MAP2 has been suggested previously by Aamodt and Williams (28) as mediating the formation of a viscous complex between neurofilaments and microtubules. They also showed that MAP preparations enriched in \(\tau\) did not promote a viscous complex formation to the same degree as the high molecular weight MAP preparations. Boiled MAP preparations also failed to promote the increase in viscosity, suggesting that boiling might alter the protein conformation. MAPs are a complex group of proteins. The current working definition includes three MAP1, two MAP2, and five \(\tau\) polypeptides (39). Which of these components bind or affect binding to neurofilaments is of great interest and under study.

In experiments using purified reconstituted filaments,
MAPs bound to reassembled NF70 and not to glial filaments. The molar ratio of binding is not affected by the presence of NF200 or NF150. These results indicate that the associated proteins are not required for binding, do not mask the binding site on NF70, and do not have cooperative effects on the binding. The 1:45 MAP:NF70 molar ratio of binding could in fact reflect the organization of the cytoskeleton in vivo. Neurofilaments organized in bundles are cross-linked to each other (6–8) and participate in more intrafilament links. Fewer sites are therefore available on the neurofilament to be linked to microtubules. The actual number of MAP molecules that are necessary to form bridges between microtubules and neurofilaments is not clear, but from the competition experiments with microtubules shown in Table I, it is clear that the binding sites for filaments are different than the sites for microtubules. These results are in agreement with recent observations of Leterrier et al. (40), which showed that MAPs bound to neurofilaments can promote microtubule assembly, as well as the viscosity experiments of Aamodt and Williams (28). In addition, NF70 filaments do not bind to microtubules in the absence of MAPs (Table I).

The studies presented in this paper show that the binding site for MAPs on the neurofilament is on NF70. We have not attempted to address the reverse question, i.e., which MAP binds to neurofilaments. Our data suggest, however, that both MAP1 and MAP2 can bind to NF70 (Fig. 6), but most of the binding studies were done using 32P-MAPs. Since MAP2 is the primary MAP protein phosphorylated under the conditions we used (Fig. 1b), the binding parameters determined reflect the binding of MAP2 to NF70. It is of course possible that τ proteins as well as MAP1 bind equally or more efficiently to NF70, but the question of the differential binding of these proteins is the subject of another study. The presence of MAP1 in the pellet when reassembled NF70 was used (Fig. 6, lanes b and d) indicates that it associates in some manner with the neurofilament. This association could be either through a complex of MAP molecules, which includes both MAP1 and MAP2, or through direct binding of MAP1 to NF70.

The specific binding of MAPs directly to the core protein of the neurofilament (NF70) suggests that MAP-mediated cross-linking between intermediate filaments and microtubules is not limited to neuronal cells, but can occur in other cell types, which do not have intermediate filament-associated proteins similar to neurofilaments. We observed weak binding of MAPs to glial filament protein and vimentin, consistent with the colocalization of MAPs to vimentin in fibroblasts (41) and significant binding to keratins. The reduced binding to glial filament protein and vimentin might be a result of the binding of components of the preparation, which are less labeled, consistent with the observation that MAP2 is abundant in neurons, and MAP1 in other cell types (42). It is possible that in regions where MAP2 is less abundant or not present, MAP1, τ, or NF200 has the role of cross-linker between intermediate filaments and microtubules.

The role that the interactions between MAPs and NF70 play in neuronal structure and physiology is not clear, but the evidence that the interactions involve the core filament protein, which precedes the appearance of the associated protein (NF200) (43, 44) in development, indicates that these interactions might have a role in the developing plastic cell as well as in the stable adult neuronal cytoskeleton. This could suggest a functional rather than structural role for these interactions. The fact that MAPs bind directly to the core filament and that the filament binding site on the MAPs is different from the tubulin binding site does not in itself indicate that a single MAP molecule is responsible for the cross-bridges in vivo. The apparent size of the cross-bridges observed by electron microscopy would imply that more than one molecule is responsible for the cross-bridges, and it is therefore even possible that these cross-bridges are composed of a complex of MAPs and other as yet unidentified cytoskeletal-associated proteins.

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