A Novel Microtubule-associated Protein from Mammalian Nerve Shows ATP-sensitive Binding to Microtubules

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Abstract. We report the isolation of a protein from mammalian nerve which shows ATP-sensitive binding to microtubules and ATPase activity. This protein, which we have designated HMW4, was prepared from bovine spinal nerve roots by microtubule affinity and ATP-induced release, and was further purified by sucrose density gradient centrifugation. It is a high molecular weight protein with a denatured Mr of 315,000, a Stokes radius of 90 Å, and a sedimentation value of ~19S. It can be resolved electrophoretically from the well-characterized bovine brain microtubule-associated proteins (MAPs) and also appears to be distinct from MAP IC. HMW4 has a vanadate-sensitive and azide-insensitive ATPase activity which averages 20 nmol Pi/min per mg protein and is different from dynein and myosin ATPases. HMW4 prepared on sucrose gradients exhibits binding to MAP-free microtubules in the absence of ATP which is reduced by ATP addition. Assayed by darkfield microscopy, HMW4 causes bundling of MAP-free microtubules which is reversed by ATP addition.

The rapid transport of membrane-bound organelles in nerve axons is a striking and easily observable example of a process common to all animal cells (16, 31). Although the identity of the cellular machinery that generates the force for rapid transport is unknown, several of its characteristics have been identified. First, a large body of physiological and morphological evidence indicates that transport occurs in association with microtubules (17, 18) probably mediated by cross-bridges between microtubules and organelles (20, 24, 34). Second, studies with permeabilized axons show that vesicle movement has an absolute dependence on the hydrolysis of ATP (1, 14). Because of these two characteristics it has been widely anticipated for some years that rapid transport might involve a cross-bridge cycle analogous to that found in muscle or cilia. Some support for the involvement of actomyosin or dynein in fast transport has come from studies of the effects of inhibitors of these mechanochemical enzymes (9, 10, 12).

In apparent contradiction to these studies, recent work with isolated cell-free systems has suggested the possibility of a novel mechanism for rapid organelle transport (3, 36). Movement of microtubules along glass surfaces and of artificial organelles along microtubules has been produced by a soluble extract of squid axoplasm and by a purified preparation from this axoplasm, the principal component of which is a 110-kD polypeptide (37). While the nature of this protein, designated kinesin, is currently under investigation, it already seems clear that it is different from both myosin and dynein. If kinesin functions in axonal transport it might work in addition to, or conceivably in conjunction with, a different mechanochemical system. In particular, there is reason to believe that the motile machinery for transport in the two directions in the axon—toward and away from the cell body—may be different (2, 13). This notion has been reinforced by the finding that kinesin generates force in just one direction along microtubules—from the minus toward the plus ends (38). Since movement in the axon proceeds in both directions, and since crude soluble extracts of axoplasm support bidirectional movement (3), it is reasonable to presume that some still unidentified factor is responsible at least for movement toward the minus ends of microtubules.

In an effort to identify proteins which might play a role in the mechanochemistry of rapid axonal transport, we have used a purification scheme which selects for proteins which have an ATP-sensitive interaction with microtubules. We have used bovine spinal nerve roots as our starting material, since this tissue is less complex than brain and is a richer source of axoplasm while still providing sufficient material for standard biochemical procedures (30). Using a microtubule affinity procedure, we have purified from bovine spinal nerve roots a high molecular weight (HMW) protein apparently distinct from the well-characterized brain microtubule-associated proteins (MAPs). This protein has microtubule-binding characteristics and ATPase activity appropriate for involvement in organelle transport.

Materials and Methods

Preparation of Microtubule Protein and HMW4

Spinal nerve roots free of their surrounding dura were obtained by dissection of fresh bovine spinal cords. They were homogenized in 1–2 vol of cold

1. Abbreviations used in this paper: HMW, high molecular weight; MAP, microtubule-associated protein.
Fractions from the preparation procedure were analyzed by SDS PAGE on 6% gels. Lanes contain 45 μg each of high speed supernatant of nerve roots (a), supernatant of microtubule pelleting (b), crude microtubule pellet (c), washed microtubule pellet (d), pellet from the ATP extraction (e), and the ATP-released supernatant (f). The positions of molecular mass standards (× 10³) are indicated by arrows at the left of the figure. The arrow at right denotes the HMW polypeptide enriched in the ATP-released supernatant.

0.1 M Pipes, 2.5 mM MgSO₄, 1 mM EGTA, pH 6.94 (PME) containing 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% protease inhibitor stock (0.2 mg/ml pepstatin A, 2 mg/ml soybean trypsin inhibitor, 2 mg/ml p-tosyl-l-arginine methyl ester, 2 mg/ml N-α-benzoyl-l-arginine methyl ester, 2 mg/ml L-1-tosylamide-phe-nylethylchloromethyl ketone, 0.2 mg/ml leupeptin). The homogenate was centrifuged at 30,000 g for 45 min, 4°C, and the supernatant was centrifuged again at 150,000 g for 90 min, 4°C. To this high speed supernatant was added 25% glycerol, 0.5 mM GTP, and 20 μM taxol (39). It was incubated at 30°C for 30 min to induce microtubule polymerization and then centrifuged at 100,000 g for 60 min, 30°C. The supernatant was discarded and the microtubule pellet was washed by resuspension in 5 vol of PME plus 20 μM taxol, 25% glycerol, and 1 mM DTT, and then centrifuged for 45 min at 100,000 g, 30°C. The washed microtubule pellet was then extracted by resuspension in 5–10 vol of PME plus glycerol, taxol, DTT, and 5 μM ATP, incubated 15 min, and then centrifuged as in the washing step. The ATP-released supernatant was dialyzed overnight at 4°C against 100 vol of PME containing 1 mM 2-mercaptoethanol and 0.1% protease inhibitor stock, and then applied to a gradient of 5–25% sucrose in the same buffer and centrifuged at 125,000 g for 6.5 h, 4°C. Fractions from the gradient were analyzed for protein content by SDS PAGE. Fractions containing HMW 4 were dialyzed at 4°C against 100 vol of PME containing 1 mM 2-mercaptoethanol and 0.1% protease inhibitor stock, and then applied to a gradient of 5–25% sucrose in the same buffer and centrifuged at 125,000 g for 6.5 h, 4°C. Fractions from the gradient were analyzed for protein content by SDS PAGE. Fractions containing HMW 4 were dialyzed at 4°C against 100 vol of PME plus 1 mM 2-mercaptoethanol. For size calibration, identical sucrose gradients were loaded with 1 mg each of tubulin (6.6S), catalase (11.3S), and thyroglobulin (19S), and centrifuged in parallel with gradients loaded with sample.

Other Biochemical Procedures

MAPs and MAP-free tubulin were prepared from twice-cycled bovine brain microtubule protein by the method of Murphy and Borisy (26), dialyzed against 100 vol of PME plus 0.1 mM GTP, and stored under liquid nitrogen. ATPase assays were performed using the method of Taylor (35). Protein determinations were made by the method of Bradford (7). Size filtration chromatography was performed using a 1 × 38-cm column of Sepharose CL-6B equilibrated in PME plus 150 mM NaCl and 1 mM 2-mercaptoethanol. The column was run at a linear flow rate of 5 cm/h and thyroglobulin, apoferritin, alcohol dehydrogenase, β-amylose, and bovine serum albumin were used as size standards. PAGE was performed by the method of Laemmli (22), using a 1.5-mm-thick gels of the following types: SDS and 6% polyacrylamide; SDS and a 3–8% exponential gradient of polyacrylamide; 2 M urea and 4% polyacrylamide; or a 1–8 M urea/3–8% polyacrylamide linear gradient. Relative molecular masses were estimated on exponential gradient gels using myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and egg albumin as standards. Proteins were stained with Coomassie Brilliant Blue R250 or with silver (25). Coomassie-stained gels were scanned to quantitate the relative amounts of protein in the bands using a Joyce Loebel Chromoscan 3 densitometer; areas under the peaks were determined by weighing.

Partial Peptide Mapping

Total MAPs from spinal nerve root microtubule preparations were obtained by suspending the microtubule pellets in 10 vol of PME containing 0.3 M NaCl, then pelleting the extracted microtubules as in the ATP extraction. Samples of the supernatant containing HMW 1–4 were then electrophoresed, along with brain MAPs, on 2 M urea/4% polyacrylamide gels (5). These were stained for 60 min in Coomassie Brilliant Blue, destained for 30 min, and the amount of protein in each band was determined by den-
Individual polypeptide bands were then excised and treated with the tryptophanyl reagent N-chlorosuccinimide (Koch-Light Laboratories, Colnbrook, England) to digest the protein by the method of Lischwe and Ochs (23; see also reference 11). After a 30-min digestion gel slices were equilibrated with a buffer containing 0.125 M Tris, pH 6.8, 10% glycerol, 3% SDS, 5 mM DTT, 0.05% bromphenol blue. Gel slices containing 3–4 μg of each digested protein were loaded into the wells of a 12% SDS polyacrylamide gel. The peptide fragments were electrophoresed and stained with silver (25).

**Pelleting Assay**

MAP-free microtubules were prepared by taxol-stimulated polymerization of pure bovine brain tubulin. Microtubules (0.7 mg/ml) were incubated with or without sucrose gradient purified HMW4 (0.1 mg/ml) for 10 min at room temperature and then sedimented for 10 min at 50,000 g in a Beckman Airfuge. The supernatants and pellets from this procedure were analyzed on 6% SDS polyacrylamide gels and the relative amounts of HMW4 and tubulin were determined by densitometry.

**Microscopy**

Darkfield observations were made using a Zeiss 50 W mercury arc, a Zeiss darkfield condensor, and a Zeiss 100x Apochromatic objective with a variable aperture. MAP-free microtubules (0.4 mg/ml) were incubated with or without sucrose gradient-purified HMW4 (0.1 mg/ml) for 10 min and then observed. A volume of ~2 μl of each incubation mixture was placed under a 22-x 22-mm coverslip for viewing. Photographs were taken using Tri X film at ASA 1600.

**Results**

**Preparation of Microtubule Protein**

By taxol treatment of high speed supernatant from 50 g of spinal nerve roots, we obtained an average of 10 mg of microtubule protein. In addition to tubulin, these pellets contained neurofilament protein, several low molecular weight polypeptides, and four HMW polypeptides with *M*_s of 315–365 kD which we have designated HMW 1–4 (Figs. 1 and 2). ATP extraction of these pellets released to the supernatant 20% of the total protein. This consisted of neurofilament protein, a small amount of tubulin and HMW2, one low molecular weight peptide, and HMW4. HMW4, with an *M*_ of 315 kD, was the HMW polypeptide most highly enriched by ATP extraction; it comprised 10% of the protein in the ATP-released supernatant, a four- to fivefold enrichment over the microtubule pellet (Figs. 1 and 2). As calculated from densitometry of gel lanes adjusted to equal protein (Fig. 2) and the distribution of total protein in the extraction procedure, ~50% of the total HMW4 in the pellets was liberated by a single ATP extraction. The other three HMW polypeptides were distributed between the extracted microtubule pellet and ATP supernatant, respectively, as follows: HMW1, 90% - 10%; HMW2, 80% - 20%; HMW3, 95% - 5%. Calculation of these values also revealed that the total amount of each HMW polypeptide did not change during the extraction procedure. Sedimentation of the ATP-extracted material on sucrose density gradients separated the neurofilament protein, tubulin, low molecular weight peptide, and HMW2 from HMW4, which sedimented at approximately the same position as thyroglobulin, giving an estimated sedimentation value of 19S (Fig. 3). When the peak HMW4 fractions were dialyzed to remove the sucrose and applied to a Sepharose CL-6B size filtration column, HMW4 eluted with a *K*_ of 0.24, giving an apparent Stokes radius of 90k and an estimated native molecular mass of 875 kD, assuming a globular conformation (Fig. 4).

**Comparison of Nerve Root HMW Polypeptides to Bovine Brain MAPS**

When compared to brain MAPs on urea-PAGE (Fig. 5, a and b), HMW4 showed a mobility between that of MAPs IC and 2A. HMW3 migrated between MAPs 1A and 1B, and HMW1 and HMW2 migrated slower than MAP 1A. To compare the brain MAPs and the two major spinal nerve root MAPs more carefully, we subjected them to digestion with the tryptophanyl reagent N-chlorosuccinimide (23). Chemical rather than enzymatic digestion was used here because the comparison of greatest interest was that between HMW4 and MAP IC, and MAP 1C is known to be highly resistant to enzymatic proteolysis (5). In addition, enzymatic cleavage of MAPs has produced conflicting results (6, 19). The partial peptide maps produced by N-chlorosuccinimide digestion (Fig. 5) revealed that HMW4 has several intermediate and low molecular weight N-chlorosuccinimide fragments not generated by digestion of the MAP1 polypeptides. Neither longer digestion (up to 15 h) nor heavier loading of the brain MAPs produced these fragments, suggesting that HMW4 is distinct from these proteins. HMW3, however, had a pattern of N-chlorosuccinimide fragments very similar to those of MAP1B and IC.

**Microtubule Binding and Bundling Activity of HMW4**

MAP-free tubulin was polymerized into microtubules by incubation with 20 μM taxol at room temperature. When these microtubules were diluted to a concentration of 0.4 mg/ml with PME and viewed in darkfield optics, they appeared as characteristic separate long filaments (Fig. 6 a). When
Figure 4. The size and molecular mass of HMW4 were determined using SDS PAGE and size filtration chromatography. In a, a plot of relative mobility versus molecular mass for standard proteins on a 3–8% exponential gradient SDS gel gives a linear correlation coefficient of \( r^2 = 0.999 \) (closed circles). The \( R_I \) of HMW4 indicates an \( M_r \) of 315 kD (closed triangle). A plot of \( K_{av} \) vs. Stokes radius for size filtration on a Sepharose CL-6B column (b) yields an estimated radius of 90 Å for HMW4 (standard proteins, closed circles; HMW4 closed triangles). Two other methods of analyzing this data gave similar estimates for Stokes radius (not shown). A plot of \( K_{av} \) vs. molecular mass (log scale) for the same column gives a linear correlation coefficient of \( r^2 = 0.993 \) for standard proteins (closed circles) and an estimated native molecular mass of 875 kD for HMW4 (closed triangle).

Figure 5. Comparison of HMW polypeptides from nerve to bovine brain MAPs. Bovine HMW MAPs (a) and HMW 1–4 (b) were resolved by PAGE on 4% acrylamide, 2 M urea gels; only the high molecular mass region is shown in a and b. Individual bands were excised, the polypeptides were digested with N-chlorosuccinimide and the fragments were separated by SDS PAGE on a 12% gel to yield partial peptide maps: MAP2A (c); MAP2B (d); MAP1A (e); MAPIB (f); MAPIC (g); HMW4 (h); and HMW3 (i).

diluted to the same concentration with sucrose gradient-purified HMW4, most of the microtubules were gathered into long thin bundles (Fig. 6 b). The addition of 1 mM MgATP to bundled preparations caused virtually complete dispersal of the bundles into individual microtubules within 2 min (Fig. 6 c). To further characterize the association of HMW4 with microtubules, we sedimented the microtubules from incubation mixtures identical to those used for the darkfield observations except that the concentration of microtubules was 0.7 mg/ml (Fig. 7). Under these conditions a 10-min spin at 50,000 g pelleted >80% of the tubulin while pelleting no detectable HMW4 in the absence of microtubules. When pellets and supernatants of this procedure were analyzed by densitometry of SDS polyacrylamide gels, 82% of the HMW4 was seen to pellet with microtubules in the absence of ATP, while 50% pelleted with microtubules in the presence of ATP.

**ATPase Activity**

The ATPase activity of HMW4 was low relative to soluble dynein or actin-activated myosin, averaging 20 nmol P/min per mg protein, but the response of the activity to different assay conditions was consistent. As shown in Table I, HMW4 MgATPase activity was insensitive to azide. It was, however, strongly inhibited by vanadate ion with half-maximal inhibition occurring at \( \sim 1 \mu \text{M} \). EHNA at 1 mM (a 10-fold excess over ATP) showed only a slight inhibition of HMW4 MgATPase activity. Although potassium could partially substitute for magnesium as a cation (45% activity), calcium could not.

**Discussion**

We have reported here the identification of a novel micro-
Figure 7. The microtubule binding behavior of sucrose gradient-purified HMW4 was examined by SDS PAGE on 6% gels. When MAP-free microtubules were incubated in PME at a concentration of 0.7 mg/ml and then pelleted, the pellets contained only tubulin (a). When microtubules were incubated at the same concentration with 0.1 mg/ml of HMW4 and then pelleted, HMW4 cosedimented with them (b). When the same assay was performed in the presence of 1 mM ATP, the amount of HMW4 cosedimenting with the microtubules was decreased (c). Densitometry of gels indicates that 82% of the HMW4 cosedimented with the microtubules in the absence of ATP, and 50% cosedimented in 1 mM ATP; in the absence of microtubules, no detectable HMW4 sedimented.

Figure 6. The microtubule bundling behavior of sucrose gradient-purified HMW4 was examined by darkfield optics. MAP-free microtubules diluted to a concentration of 0.4 mg/ml with PME and viewed in darkfield optics appear as separate filaments (a). When microtubules are diluted to the same concentration with HMW4 in PME at a concentration of 0.1 mg/ml, most of the microtubules are seen in darkfield to be gathered into bright bundles which can be clearly distinguished from individual microtubules in darkfield (b). When the mixture shown in b was incubated with 1 mM ATP, most of the microtubules were released from the bundles (c). Bar, 15 μm.

Table I. ATPase Activity of HMW4 under Different Conditions*

<table>
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<tr>
<th>Assay conditions</th>
<th>% Activity</th>
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<tr>
<td>5 mM MgSO4</td>
<td>100</td>
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<tr>
<td>+ 10 μM azide</td>
<td>99</td>
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<tr>
<td>+ 1 mM vanadate</td>
<td>53</td>
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<td>+ 10 μM vanadate</td>
<td>19</td>
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<tr>
<td>+ 100 μM vanadate</td>
<td>0</td>
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<tr>
<td>+ 1 mM EHNA</td>
<td>82</td>
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<td>K⁺/EDTA</td>
<td>45</td>
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<td>5 mM CaCl₂</td>
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*ATPase activity was measured in 100 mM Tris-HCl, 200 mM NaCl, 100 μM ATP, pH 8.0, at 25°C. The average specific activity was 20 nmol P/min per mg protein.

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the calcium-stimulated ATPase activity and EHNA sensitivity characteristic of dynesins (29). Its insensitivity to azide indicates that HMW4 is different from the microtubule-associated ATPase similar to mitochondrial F1 ATPase found in mammalian nervous tissue (27).

The most unusual property of HMW4 is its ATP-sensitive binding and bundling of microtubules. The possibility exists that the microtubule binding and ATP-dependent release are actually properties of other proteins in the crude microtubule pellets, with which HMW4 interacts passively. This is made unlikely by the fact that ATP extraction specifically enriches for HMW4 more than for any other protein in the supernatant. And the pelleting assays performed with MAP-free microtubules and pure HMW4 rule this out; in the virtual absence of any other proteins, HMW4 still binds to and pellets with microtubules, and the percentage of it which does so is reduced from 82% to 50% by ATP addition. This 50% ATP-insensitive binding is similar to that of the crude microtubule pellets in the preparation procedure. The ATP-dependent release is likely to involve ATP binding and/or hydrolysis by HMW4 rather than a phosphorylation event, since attempts to demonstrate phosphorylation of HMW4 under the conditions of the ATP extraction assay have been negative (data not shown).

That HMW4 can bundle microtubules suggests that the molecule has at least two sites for binding. The release of microtubule bundling by the addition of ATP must be due to either the complete dissociation of HMW4 from the microtubules or the release of one site of binding. Under the conditions of the pelleting assay, ~40% of the HMW4 bound is completely released from microtubule binding by ATP addition. Either this reduction in the amount of HMW4 bound to the microtubules is sufficient to release them from bundles, or the HMW4 molecules remaining bound are attached to only one microtubule. The former possibility seems unlikely, since HMW4 will bundle microtubules at lower HMW4/tubulin ratios than shown in Fig. 6. The data are thus consistent with the notion that the binding and bundling properties of HMW4 are due to two different binding sites on the molecule, one ATP sensitive and one ATP insensitive.

Several characteristics make HMW4 a reasonable candidate for involvement in the mechanochemistry of rapid axonal transport. Regardless of the precise identity of the motor driving organelle movement, we can infer from physiological studies that it probably interacts with microtubules in a transient fashion coupled in some way to the binding and hydrolysis of ATP. The binding of HMW4 to microtubules in the absence of ATP, and release in the presence of ATP are consistent with its participation in a cycle of microtubule-associated force generation coupled to ATP binding and hydrolysis. The ATP-insensitive binding may reflect a specific affinity for microtubules or a more general binding to an appropriate surface; studies with artificial organelles suggest that the antero- or retrograde transport motor can attach to any surface with the appropriate charge and move it relative to microtubules (2).

The ATPase activity of HMW4 shares with retrograde organelle transport a vanadate sensitivity (38). Although the specific ATPase activity of HMW4 is considerably lower than that of dynein or myosin, this may be an artefact of preparation or a consequence of measuring its activity in the soluble and unbound form. In addition, the recent in vitro studies with kinesin suggest that force generation may be accomplished by enzymes with low specific ATPase activity similar to that of HMW4 (37).

Lastly, ultrastructural studies of axons using a variety of fixation techniques all reveal cross-bridges between microtubules and organelles as well as other microtubules. If, as is likely, at least some of these structures represent the motor driving organelle transport, then that motor must be sufficiently large to constitute a cross-bridge of ~17 nm (24). The large Stokes radius and sedimentation value of HMW4 suggest that it is large enough to meet this requirement. We are currently investigating whether HMW4 is capable of generating movement in vitro.

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