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Abstract
Neurofilaments are synthesized in the cell body of neurons and transported outward along the axon via slow axonal transport. Direct observation of neurofilaments trafficking in live cells suggests that the slow outward rate of transport is due to the net effects of anterograde and retrograde microtubule motors pulling in opposition. Previous studies have suggested that cytoplasmic dynein is required for efficient neurofilament transport. In this study, we examine the interaction of neurofilaments with cytoplasmic dynein. We used fluid tapping mode atomic force microscopy to visualize single neurofilaments, microtubules, dynein/dynactin, and physical interactions between these neuronal components. AFM images suggest that neurofilaments act as cargo for dynein, associating with the base of the motor complex. Yeast two-hybrid and affinity chromatography assays confirm this hypothesis, indicating that neurofilament subunit M binds directly to dynein IC. This interaction is blocked by monoclonal antibodies directed either to NF-M or to dynein. Together these data suggest that a specific interaction between neurofilament subunit M and cytoplasmic dynein is involved in the saltatory bidirectional motility of neurofilaments undergoing axonal transport in the neuron.

Comments

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The Interaction of Neurofilaments with the Microtubule Motor Cytoplasmic Dynein

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Neurofilaments are synthesized in the cell body of neurons and transported outward along the axon via slow axonal transport. Direct observation of neurofilaments trafficking in live cells suggests that the slow outward rate of transport is due to the net effects of anterograde and retrograde microtubule motors pulling in opposition. Previous studies have suggested that cytoplasmic dynein is required for efficient neurofilament transport. In this study, we examine the interaction of neurofilaments with cytoplasmic dynein. We used fluid tapping mode atomic force microscopy to visualize single neurofilaments, microtubules, dynein/dynactin, and physical interactions between these neuronal components. AFM images suggest that neurofilaments act as cargo for dynein, associating with the base of the motor complex. Yeast two-hybrid and affinity chromatography assays confirm this hypothesis, indicating that neurofilament subunit M binds directly to dynein IC. This interaction is blocked by monoclonal antibodies directed either to NF-M or to dynein. Together these data suggest that a specific interaction between neurofilament subunit M and cytoplasmic dynein is involved in the saltatory bidirectional motility of neurofilaments undergoing axonal transport in the neuron.

INTRODUCTION

Neurofilaments (NFs) are neuron-specific intermediate filaments composed of three subunits: neurofilament light chain (NF-L), medium chain (NF-M), and heavy chain (NF-H). These subunits assemble as heteropolymers that provide structural support to the axon. NFs are synthesized in neuronal cell bodies and are then moved outward along the axon via slow transport along microtubules, moving at rates of ~0.1–3 mm/d. Direct observations of the motility of labeled neurofilaments moving along the axons of cultured neurons indicate that this overall slow axonal transport is the net effect of rapid, intermittent, and highly asynchronous bidirectional motility interrupted by prolonged pauses (Prahlad et al., 2000; Roy et al., 2000; Wang et al., 2000). Although both the outward and inward movements of the neurofilaments are intermittent and of short duration, the rates of anterograde and retrograde motility are consistent with the transport rates of the microtubule motor proteins kinesin and cytoplasmic dynein (Roy et al., 2000; Wang et al., 2000). Therefore, the fast axonal transport motors kinesin and dynein are believed to be involved in transporting neurofilaments as heteropolymers (reviewed in Shea and Flanagan, 2001). Defects in this transport may contribute to the aggregations of neurofilaments observed in several genetically unrelated neurodegenerative diseases, including ALS and Charcot-Marie-Tooth disease (reviewed in Al-chalabi and Miller, 2003).

The bidirectional motility of neurofilaments along microtubules has been reconstituted in vitro (Shah et al., 2000). Both conventional (Prahlad et al., 2000; Xia et al., 2003) and unconventional kinesins (Shah et al., 2000) may drive the anterograde motility of neurofilaments. The mechanism by which kinesin or a kinesin-like protein interacts with neurofilaments has not yet been examined.

Several observations indicate that cytoplasmic dynein is the motor for the minus-end directed motility of neurofilaments along microtubules observed both in vivo and in vitro. Neurofilaments accumulate in the axons of transgenic mice with a targeted disruption of the dynein-activator complex, dynactin, suggesting that retrograde trafficking mediated by dynein is critical for neurofilament transport (LaMonte et al., 2002). Further, dynein accumulates in neurofilament-rich aggregates formed in axonal swellings after administration of beta, beta'-iminodipropionitrile (IDPN; Toyoshima et al., 1998). Cytoplasmic dynein copurifies with neurofilaments from spinal cord, and antibodies directed against dynein IC (DIC) decorate purified native neurofilament polymers. Antidynein antibodies also were found to inhibit the motility of neurofilaments along microtubules in vitro (Shah et al., 2000).

Here we analyze the interaction between cytoplasmic dynein and dynactin, neurofilaments, and microtubules in greater detail. We used atomic force microscopy to image the microtubule-neurofilament and dynein/dynactin-neurofilament interactions in unfixed, unstained specimens, in comparison to electron micrographs of similar preparations. We also examined the dynein-neurofilament interaction biochemically and identified a direct interaction between neurofilament subunit M and the IC of cytoplasmic dynein. Together, these results support a role for cytoplasmic dynein in the short-duration retrograde movement of neurofila-


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ments undergoing slow anterograde transport along the microtubules of the axon.

**MATERIALS AND METHODS**

**Protein Purification and Labeling**

NFs were isolated from bovine spinal cords as previously described (Leterrier et al., 1996). The purity of neurofilament preparations was routinely checked by densiometry of Coomassie-stained gels after SDS-PAGE. For the preparations used in these studies the neurofilament triplet subunits (NF-H, NF-M, and NF-L) accounted for >95% of the total protein (see Results). The neurofilament suspension was stored in aliquots at ~70°C after rapid freezing in liquid nitrogen; protein concentrations were determined using the Lowry assay (Lowry et al., 1951). Neurofilaments were fragmented for some studies using a Sonic Dismembrator 60 (Fisher Scientific, Pittsburgh, PA) on diluted neurofilament samples (15–30 μg/ml) in reassembly buffer: RB (0.1 M Mes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) on ice, using a 1-s burst at a setting of 5W for 3–5 s total. Neurofilaments were dephosphorylated for some studies by incubation with *Escherichia coli* alkaline phosphatase (Sigma, St. Louis, MO) at 3 μg/nF for 72 hr under dialysis at 4°C against RB buffer with 0.8 M sucrose and 1 mM PMSF (phenylmethylsulfonyl fluoride).

The dynein/dynactin complex was isolated from rat brain by microtubule affinity and ATP extraction, followed by purification by sedimentation through a sucrose gradient (Karki and Holzbaur, 1995). The peak 19–20S affinity and ATP extraction, followed by purification by sedimentation with DIC. The majority of clones isolated were found to encode p150Glu, and we further screened positives by Southern blot to identify novel clones, one of which was identified by database searching as encoding a fragment of NF-M-spanning residues 122–428. Full-length clones encoding NF-M and NF-L were generously provided by Dr. Virginia Lee of the University of Pennsylvania. A subclone of NF-L–spanning residues 100–420 was generated by restriction digests and PCR. Both the full-length and partial clones of NF-M and NF-L were tested in bidirectional transport assays with DIC; the results were scored qualitatively in comparison to both positive and negative controls.

**Affinity Chromatography and Cosedimentation Binding Assays**

Affinity matrices were generated by covalently coupling purified recombinant dynein IC to Sepharose beads, as previously described (Karki and Holzbaur, 1995). A recombinant DIC construct (pETl35bDIC) was expressed in the BL21 strain of *E. coli*, and the His-tagged protein was purified by nickel sulfate chromatography. The purified protein was covalently coupled to activated CH-Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ). The TNT T7 Quick coupled transcription/translation system (Promega, Madison WI) was used to express full-length cDNAs encoding NF-M and NF-L, and subclones spanning residues 122–428 of NF-M and residues 100–420 NF-L in vitro. The resulting proteins were then loaded onto the rDIC affinity matrix and batch-bound with gentle shaking at 25°C for 10 min. After extensive washing with phosphate-buffered saline and 0.2% Triton X-100, the columns were eluted by boiling the beads in sample buffer for 5 min and collecting the supernatant. The resulting fractions were analyzed by SDS-PAGE and autoradiography.

**RESULTS**

The binding of endogenous neurofilaments to DIC was examined by incubating a rat brain homogenate and centrifugation at 39,000 × g, batchwise with rotation with either DIC or BSA-bound Sepharose beads. After extensive washes with HEM buffer (100 mM HEPES, 1 mM EGTA, 1 mM MgSO₄, pH 7.3) with 25 mM NaCl and 0.1% Triton X-100, bound proteins were eluted by heating the beads in sample buffer for 5 min and collecting the supernatant. Gel bands resulting from the supernatant and pellet fractions were analyzed by SDS-PAGE and Western blot.

Cosedimentation experiments were performed with sonicated neurofilaments, both phosphorylated and dephosphorylated, dynein/dynactin, and microtubules stabilized with taxol. After a 30-min incubation at room temperature, binding reactions were centrifuged for 20 min at 17,000 × g. To test the nucleotide dependence of the association, MgATP was added to some reactions at 5 mM at the start of the incubation, and an additional 5 mM MgATP was added before centrifugation to replenish ADP due to the activity of the dynein ATPase. Gel samples from the resulting supernatant and pellet fractions were analyzed by SDS-PAGE and Western blot.

**Electron Microscopy of Neurofilament-Microtubule Complexes**

Purified NFs were incubated for 30 min with assembly competent reconstituted tubulin (isolated from the co-cycled microtubules by phenylisocyanate chromatography) in RB with 1 mM GTP. Samples were loaded on glow-discharged formvar-carbon–coated 300 mesh copper grids, stained with 1% uranyl acetate in H₂O for 20 s, dried in air after side adsorption of the drop, and observed in a Jeol 100 electron microscope (Peabody, MA) at 80 kV.

**Yeast Two-Hybrid Interaction Screen**

We used the LexA yeast two-hybrid system to screen a random-insert human cDNA library (Stratagene, La Jolla, CA) for proteins interacting with DIC. The majority of clones isolated were found to encode p150Glu, and we further screened positives by Southern blot to identify novel clones, one of which was identified by database searching as encoding a fragment of NF-M-spanning residues 122–428. Full-length clones encoding NF-M and NF-L were generously provided by Dr. Virginia Lee of the University of Pennsylvania. A subclone of NF-L–spanning residues 100–420 was generated by restriction digests and PCR. Both the full-length and partial clones of NF-M and NF-L were tested in bidirectional transport assays with DIC; the results were scored qualitatively in comparison to both positive and negative controls.

**Fluid phase tapping mode AFM was used to visualize single neurofilaments, microtubules, and their interactions.**
expectation for filaments undergoing Brownian motion being deposited from solution onto an adhesive surface (Wagner et al., 2003; Dogic et al., 2004). Microtubules, in contrast, are easily differentiated from NFs because they are wider and much stiffer than neurofilaments (Figure 1, c–f). Height measurements reveal an average NF height of 8–10 nm compared with 25 nm for MTs (Figure 1g).

When mixtures of microtubules and neurofilaments are imaged (Figure 1, c–f), there are numerous interactions between the polymers, but it is not possible to discriminate biochemical bonds from steric interactions between the filaments. Frequently observed structural features include neurofilaments that appear to be draped over the larger microtubules (small arrow in Figure 1d) and sites where neurofilaments appear to be adjacent to but not in contact with microtubules. The latter feature is seen in more detail in Figure 1, e and f, which shows that even with the added apparent width of the filaments due to the finite size of the AFM tip, there is a clear separation between the two filament cores, possibly mediated by the neurofilament sidearms, which we cannot resolve by AFM in fluid measurements. Such close contacts for long distances between neurofilaments and microtubules are observed instead by negative staining electron microscopy of microtubules polymerized from pure tubulin in the presence of neurofilaments (Figure 2). The constant spacing between neurofilament and microtubule polymers under these conditions is 4–7 nm. The occurrence of cross-bridges between the two polymers is suggested by the direct binding of neurofilament extensions to the microtubule wall (Figure 2b).

**Cytoplasmic Dynein/Dynactin Binds to Neurofilaments**

Cytoplasmic dynein and dynactin, isolated from rat brain by microtubule affinity and ATP-release (Karki and Holzbaur, 1995), was imaged by AFM. As shown in Figure 3a, we noted a heterogeneous population. Dynactin is most easily identified in these unfixed, unstained populations due to the distinctive structure of the Arp1 filament at the base of the molecule (Figure 3, b and c; Schafer et al., 1994). The p150Glued sidearm of dynactin is also clearly visible. The orientation and morphology of cytoplasmic dynein appears to be more variable in AFM images (Figure 3d), suggesting that the molecule may be more flexible in solution and therefore may not uniformly orient on the mica surface. However, large bilobed structures are apparent, consistent with the morphology and dimensions of cytoplasmic dynein as determined by STEM (Vallee et al., 1988). Some images are
consistent with the overall morphology of a dynein/dynactin cocomplex (Figure 3e).

To examine the interaction of dynein and dynactin with neurofilaments, AFM was used to determine if binding of dynein/dynactin to purified neurofilaments could be visualized and if the motor complex increased the association of neurofilaments with microtubules. When added to purified neurofilaments, dynein/dynactin decorated the neurofilaments along their contour (Figure 4a and b) often forming thin projections of length 50–100 nm extending from the neurofilament core. The contour of the neurofilament becomes highly nonuniform, as shown by the height map of the neurofilament in Figure 4e. The additional mass on the neurofilaments and the structures extending away from their cores are likely due to binding of dynein/dynactin to multiple sites along the neurofilament. This binding was not observed if the dynein/dynactin was first incubated with an antibody to the dynein IC (Figure 4c and d). After addition of the anti-DIC antibody, the neurofilament contours were again smooth, despite the abundance of material presumed to be dynein/antibody complexes in the background. Close inspection of these images shows that the zone within 50–100 nm of the NF core is depleted of the protein complexes consistent with the overall morphology of a dynein/dynactin cocomplex (Figure 4e).

The observations of neurofilament motility along microtubules are visible farther away (Figure 4c), consistent with the hypothesis that NF-M is the primary ligand for the motor and that the movement of neurofilaments on microtubules is disrupted by the anti-DIC antibody (Shah et al., 2000).

Purified dynein/dynactin also bound to microtubules, decorating their surface in AFM images (Figure 6). The appearance of the structures that decorate the microtubules differ from those decorating neurofilaments in that the dynein/dynactin appear as a more compact mass on microtubules rather than one that extends a projection away from the neurofilament core (Figure 6a–c). No binding of dynein/dynactin to microtubules was observed in the presence of MgATP (Figure 6d).

Cytoplasmic Dynein Binds Directly to Neurofilament Subunit M

The observations of neurofilament motility along microtubules observed by Shah et al. (2000) as well as the AFM images shown above suggested to us that neurofilaments are a cargo for cytoplasmic dynein. We hypothesized that neurofilaments bind to the cargo-binding domain at the base of the motor complex. This association is likely to involve the IC of cytoplasmic dynein, because incubation of neurofilaments with anti-DIC antibodies significantly depletes bound dynein from neurofilaments (Shah et al., 2000; and Figure 4c and d). The association could either be a direct interaction, or an indirect interaction via dynactin, which binds to DIC (Karki et al., 1995), or other neurofilament-associated proteins such as NUDEL (Nguyen et al., 2004).

To examine the possibility of a direct interaction, we used a yeast two-hybrid assay to identify DIC-binding proteins from a human brain cDNA library. In the screen, we isolated a subclone of NF-M, suggesting that NF-M is a dynine-binding protein. The subclone spans residues 122–428, encoding part of the neurofilament rod domain (Figure 7). To confirm this interaction, we expressed the NF-M fragment in an in vitro transcription/translation assay and examined the association of the protein with recombinant dynein IC covalently bound to Sepharose beads using affinity chroma-
As shown in Figure 7, we observed a robust association of this domain of NF-M with DIC. In parallel experiments with full-length NF-M, we did not observe as robust binding to dynein either in yeast two-hybrid or affinity chromatography assays (Figure 7). This observation suggested to us that there may be epitope-masking in the full-length NF-M polypeptide that blocks the dynein-binding site.

To test the specificity of the interaction detected between NF-M and dynein, we subcloned the homologous region from NF-L and tested the binding of this domain (residues 100–420) as well as of full-length NF-L to dynein IC using both the yeast two-hybrid assay and affinity chromatography. As shown in Figure 7, we observed no significant interaction between the NF-L subfragment and DIC and only a minor interaction between full-length NF-L and DIC in both of these assays. However, we have noted direct interactions between NF-L and cytoplasmic dynein in blot overlay assays (our unpublished results), suggesting that while the interaction between dynein and NF-M is most robust, dynein may interact directly with assembled neurofilaments via multiple binding determinants.

**Figure 4.** AFM images of cytoplasmic dynein/dynactin complex bound to neurofilaments. (a) Dynein/dynactin decorate neurofilaments along their lengths, often forming thin projections of length 50–100 nm extending from the neurofilament core. (b) The binding of dynein/dynactin to neurofilaments occurs primarily (80%) with globular domains projecting away from the backbone of the NF, connected via a filamentous structure, probably the dynein-stem (white arrows in b). (c and d) No decoration of neurofilaments is observed in the presence of an antibody to the dynein IC. A clear exclusion zone is seen along the NF-backbone in lower resolution images. (e) Dynein/dynactin decorate neurofilaments along their lengths, so that the contour of the neurofilament becomes highly nonuniform, as shown by height maps. (f and g) Sonicated neurofilament fragments bind along the side of microtubules in the presence of dynein/dynactin. In the absence of dynein/dynactin less binding of sonicated NF fragments to microtubules is seen (our unpublished results).

**Figure 5.** Fluctuations in filament height from binding of dynein/dynactin complex. (a) Apparent height of neurofilament derived from AFM measurement along 35 μm of filament contour derived from measurements of multiple neurofilaments in different fields of the AFM image for two representative NF preparations: NF with added dynein/dynactin complex or the same preparation in the presence of an anti-DIC antibody. (b) Comparison of the number of height fluctuations per 35 μm NF contour length greater than 15 nm for neurofilaments alone, neurofilaments with added dynein/dynactin or neurofilaments with dynein/dynactin and antibodies against dynein IC (DIC), the p150Glued subunit of dynactin, NF-L, or NF-M/NF-H (SMI 31).

Both the yeast two-hybrid and affinity chromatography assays demonstrated direct binding of dynein IC to a fragment of NF-M, but not to full-length NF-M. This result suggests that the dynein binding site may be masked in the full-length protein. However, AFM images reveal the association of dynein with native neurofilament polymers and in vitro studies have shown dynein-dependent motility of neurofilaments along microtubules. Therefore we sought to test whether the observed epitope masking is relieved when NF-M becomes incorporated into polymer. To test this, we examined the binding of endogenous neurofilaments to recombinant dynein IC bound to Sepharose beads. As shown in Figure 8, when a soluble rat brain extract was incubated with DIC-bound beads, we observed the binding of NF-M to the affinity matrix. Both NF-H and NF-L were also bound to the DIC beads, although the binding of NF-H appeared less robust than that of NF-M and NF-L. In contrast, no signifi-
cant binding was observed to BSA-bound beads, demonstrating that the interactions were specific and not due to trapping of neurofilaments on the matrix. These results suggest that the assembly of NF-M into polymer may unmask the binding site for cytoplasmic dynein.

To further investigate the role of dynein in mediating interactions between neurofilaments and microtubules, we incubated sonicated neurofilaments with microtubules in the absence and presence of exogenous dynein and dynactin and then examined the cosedimentation of neurofilaments with microtubules. We performed this experiment with neurofilaments isolated from an adult animal, which are extensively phosphorylated, and also with neurofilaments that had been dephosphorylated in vitro. As shown in Figure 9A, we did not observe any significant association of phosphorylated neurofilaments with microtubules in this assay. The dephosphorylated neurofilaments also remained in the supernatant fraction in the absence or presence of exogenous dynein and dynactin. However, in the presence of microtubules we observed the specific cosedimentation of dephosphorylated neurofilaments with microtubules. A similar dephosphorylation-induced interaction between neurofilaments and microtubules was observed by Hisanaga and Hirokawa (1990) and attributed to the direct binding of dephosphorylated NF-H sidearms to the microtubule. However, as shown in Figure 9B, the association of the sonicated and dephosphorylated neurofilaments with microtubules was ATP-dependent—no significant cosedimentation was observed in the presence of Mg-ATP. The nucleotide dependence of this interaction suggests that endogenous microtubule motors may also be mediating the association between neurofilaments and microtubules. Addition of exogenous purified dynein/dynactin marginally enhanced the cosedimentation. The limited enhancement observed may be due to the low molarity of the purified dynein/dynactin (0.03 mg/ml), in contrast to the higher concentrations of neurofilaments (0.25 mg/ml) used in this assay. Alternatively, a high-affinity interaction between the motor complex and neurofilaments may require additional factors such as NUDEL (Nguyen et al., 2004).

DISCUSSION

Direct observations of neurofilament motility within neurons has shown that the characteristic slow anterograde...
movement of these filaments is the net result of rapid movements in both the anterograde and retrograde direction, interrupted by pauses (Roy et al., 2000; Wang et al., 2000). The rates of active movement in the anterograde and retrograde directions are consistent with the known motility rates of kinesin and cytoplasmic dynein, respectively.

In vitro, purified neurofilaments are translocated bidirectionally in an ATP-dependent manner along microtubules at rates of ~0.5 μm/s; microtubule minus end-directed movements are inhibited by antidynein antibodies (Shah et al., 2000).

To further investigate the role of cytoplasmic dynein and its accessory complex dynactin in mediating the motility of neurofilaments along microtubules, we examined interactions among these proteins using EM and AFM. Both approaches suggest that there are distinct points of contact between neurofilaments and microtubules. We hypothesize that microtubule motors may mediate some of these lateral contacts, allowing for the movement of neurofilaments along axonal microtubules. In vitro binding studies support this hypothesis, because we noted the ATP-dependent cosedimentation of sonicated neurofilaments with microtubules. Cosedimentation in the absence of nucleotide was enhanced by the dephosphorylation of neurofilaments, as previously noted (Hisanaga and Hirokawa, 1990). Both motor-mediated and NF-H sidearm-mediated cross-bridging may be more effective at lower levels of neurofilament phosphorylation. In vivo, the interaction of neurofilaments with microtubules is likely to be more complex, involving structural MAPs as well (Heimann et al., 1985; Hirokawa et al., 1988). Potentially, the phosphorylation-dependent NF-H sidearm-mediated interactions between neurofilaments and microtubules may be involved in the intermittent pausing noted between bidirectional motor-mediated excursions observed for neurofilaments undergoing axonal transport in situ (Roy et al., 2000; Wang et al., 2000).

AFM images suggest that most of the mass of dynein bound along neurofilaments is projecting away from the polymer. Cytoplasmic dynein is composed of two large globular heads, each ~13 nm in diameter, which bind in an ATP-dependent manner to microtubules via stalk domains (Gee et al., 1997). The large heads are each connected via relatively slender stems ~25 nm long to a common base (Burgess et al., 2003). Although the dynein heads are required for motor function, the base of the molecule is involved in attachment to cargo. Because neurofilaments act as cargo for dynein, we hypothesized that they are associated with the DIC at the base of the motor. Dynein intermediate and light chains form the cargo binding domain and have been shown to interact either directly or indirectly with specific cargos (reviewed in Karki and Holzbaur, 1999).

Here we demonstrate that neurofilaments bind directly to dynein, via an interaction between NF-M and DIC. Both yeast two-hybrid and affinity chromatography experiments demonstrate that there is a binding site for dynein in the rod domain of a neurofilament subunit. The mapping studies we have done identify the key binding site for cytoplasmic dynein within amino acid residues 122–428 of NF-M. This observation of the binding of dynein to the central domain of NF-M rather than to the tail domain is consistent with the results of Rao et al. (2003), who noted that knock-in mice expressing a tail-deleted construct of NF-M showed no alteration in rates of axonal transport of neurofilaments. Interestingly, in vitro binding studies indicate that the tail domain may actually block dynein binding to unassembled neurofilaments, because we did not observe significant binding of DIC to full-length NF-M in the absence of other neurofilament subunits. However, we did observe binding when NF-M is copolymerized with NF-L and NF-H into native neurofilaments (Figure 8), suggesting that when NF-M becomes incorporated into polymer the binding site for dynein is exposed. Although these data suggest that NF-M is a key determinant for dynein binding, we cannot rule out additional interactions between neurofilaments and cytoplasmic dynein. Although yeast two-hybrid and affinity chromatography experiments revealed only weak binding between DIC and NF-L, and AFM data indicate that an antibody to NF-M (and NF-H) disrupts dynein binding to neurofilaments more efficiently than an antibody directed to NF-L, we did note evidence for an additional interaction between DIC and NF-L using a blot overlay assay (our unpublished results). There may be multiple binding deter-
minants for the interaction between dynein and neurofilaments in vivo.

The direct interactions that we have noted between NF-M and cytoplasmic DIC also indicate that no intermediary adaptor proteins may be necessary to mediate the binding. The DIC polypeptide is localized at the base of dynein and has been shown to participate directly in some motor-cargo binding interactions. Both casein kinase II (Karpi et al., 1997) and β-catenin (Ligon et al., 2000) bind directly to DIC. In contrast, other dynein cargos may associate indirectly with dynein, via dynactin (Holleran et al., 2001). Studies on the role of dynein and dynactin in the intracellular motility of vimentin have suggested that dynactin is key in mediating cargo-binding interactions (Helfand et al., 2002). Dynactin copurifies with neurofilaments from spinal cord (Shah et al., 2000), but is likely to bind independently to neurofilaments because incubation of neurofilaments with antidynein antibodies effectively removes dynein but not dynactin from the filaments (Shah et al., 2000). We cannot rule out a possible role for dynactin in increasing either the affinity or the specificity of the interaction of the motor complex with neurofilaments. However, antidynein antibodies block the dynein/neurofilament association more effectively than antidynactin antibodies (Figures 4 and 5, and our unpublished results).

Our observations that dynein binds directly to neurofilaments and thus is the likely motor providing microtubule minus end–directed force on neurofilaments in the axon is also consistent with in vivo observations. We have noted that neurofilaments accumulate in the axons of transgenic mice with a targeted disruption in dynein/dynactin function (LaMonte et al., 2002). The microtubule plus end–directed motor is likely to be either conventional kinesin or a member of the kinesin superfamily. Although conventional kinesin was not observed to copurify with neurofilament preparations from spinal cord, Shah et al. (2000) did find evidence for the copurification of kinesin-like proteins. However, gene-targeting of KIF5A, which encodes a neuronally expressed isoform of conventional kinesin, was found to lead to the accumulation of NF-H, NF-M, and NF-L within the cell bodies of peripheral sensory neurons (Xia et al., 2003), suggesting that this kinesin may be directly involved in the outward transport of neurofilaments in vivo (Phralad et al., 2000).

The somewhat unusual tug-of-war mechanism that results in the net slow transport outward movement of neurofilaments may be required because of the intrinsic properties of neurofilament polymers. In vitro studies of the motility of neurofilaments in the axon is also consistent with in vivo observations. We have noted that neurofilaments accumulate in the axons of transgenic mice with a targeted disruption in dynein/dynactin function (LaMonte et al., 2002). The microtubule plus end–directed motor is likely to be either conventional kinesin or a member of the kinesin superfamily. Although conventional kinesin was not observed to copurify with neurofilament preparations from spinal cord, Shah et al. (2000) did find evidence for the copurification of kinesin-like proteins. However, gene-targeting of KIF5A, which encodes a neuronally expressed isoform of conventional kinesin, was found to lead to the accumulation of NF-H, NF-M, and NF-L within the cell bodies of peripheral sensory neurons (Xia et al., 2003), suggesting that this kinesin may be directly involved in the outward transport of neurofilaments in vivo (Phralad et al., 2000).

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