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Fast transport of neurofilament protein along microtubules in squid axoplasm

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SUMMARY

Using squid axoplasm as a model system, we have visualized the fast transport of non-filamentous neurofilament protein particles along axonal microtubules. This transport occurs at speeds of 0.5-1.0 μm/second and the majority of neurofilament particles stain with kinesin antibody. These observations demonstrate, for the first time, that fast (0.5-1.0 μm/second) transport of neurofilament proteins occurs along microtubules. In addition, our studies suggest that neurofilament protein can be transported as non-membrane bound, nonfilamentous subunits along axons, and that the transport is kinesin-dependent. Microtubule-based fast transport might therefore provide a mechanism for the distribution and turnover of neurofilament, and perhaps other cytoskeletal proteins, throughout neurons.

Key words: Neurofilament, Intermediate filament, Axonal transport, Microtubule, Kinesin, Cytoskeleton

INTRODUCTION

The transport of cytoskeletal components along axons, studied primarily by following the anterograde movement of 35S-labeled protein, was thought to occur at the slow rate of 0.1-4 mm/day (0.001-0.05 μm/second; Lasek et al., 1984; Galbraith et al., 1999). On the other hand, membranous organelles including synaptic vesicles and plasma membrane components were shown, using similar techniques, to move at rates corresponding to fast axonal transport (50 mm-400 mm/day; approx. 0.5-4 μm/second). Recently it was discovered that filamentous forms of GFP-tagged neurofilament protein (NIF), a Type IV intermediate filament (IF) protein, vimentin, is transported as particles (vimentin dots; vimentin particles) at rates of 0.5-1.0 μm/second along microtubules through their association with kinesin-II (Cole et al., 1998). The common features of the transport of protein complexes for structures as diverse as the axoneme of the Chlamydomonas flagellum, and the IF network of fibroblasts, led us to explore whether a similar microtubule- and kinesin-dependent mechanism might be responsible for the fast axonal transport of neurofilament proteins in axons. We have therefore attempted to determine whether Type IV IF (neurofilament) proteins, like their Type III vimentin IF counterparts (Prahlad et al., 1998), can also be transported along microtubules in the form of non-filamentous precursors. To this end, we have studied axoplasm extruded from the giant axon of the squid Loligo paelei. This is an excellent model system to resolve motility along single microtubules, and has been widely used to observe the microtubule-dependent fast transport of membrane-bound organelles (Brady et al., 1982; Allen et al., 1985; Vale et al., 1985).

MATERIALS AND METHODS

Immunofluorescence microscopy of the squid axon

Medium- to large-size squids (Loligo paelei) were obtained at
the Marine Biological Laboratory’s Marine Resources Center. Approximately 4 cm lengths of giant axons were dissected according to Weiss et al. (1991). The axons were then cut into sections of approx. 2 cm lengths for use in our studies.

In order to localize neurofilaments in squid axons, sections of dissected axons were mounted on glass coverslips, fixed for 10 minutes in 1% glutaraldehyde diluted into Buffer X (350 mM potassium aspartate, 130 mM taurine, 70 mM betaine, 50 mM glycine, 20 mM Hepes, pH 7.2, adjusted with KOH, 12.9 mM MgCl₂, 10 mM K-EGTA, 3 mM CaCl₂, 1 mM glucose, 1 mM ATP; Brady et al., 1993), permeabilized for 5 minutes using 1% Triton-X-100, and then processed for indirect immunofluorescence. Two polyclonal antibodies, one directed against squid NIF220 and the other against both squid NIFs 60 and 70 (a gift of Dr Harish Pant, NIH; Grant et al., 1995), were used as the primary antibodies. Fluorescein-labeled IgG was used as the secondary antibody (Jackson ImmunoRes. Lab. Inc., West Grove, PA, USA). Sections of the axon were incubated for 2 hours in the primary antibody and 1 hour in the secondary antibody at 37°C, in order to achieve maximal penetration into the fixed axons.

Immunofluorescence microscopy of extruded axoplasm
For studies involving axoplasm, approximately 5 μl of axoplasm was extruded from an axon into approx. 50 μl of Buffer X, according to the procedure of Brady (1993). Briefly, the samples of axoplasm were allowed to incubate in Buffer X for 10-30 minutes at room temperature, and subsequently fixed with 1% glutaraldehyde in Buffer X, stained with the appropriate antibodies and observed using a Zeiss LSM510 confocal microscope. NIF proteins within the axoplasm were localized using the polyclonal antibodies described above and a monoclonal antibody directed against phosphorylated NIF220 (SMI 31; Veeranna et al., 1995). Microtubules were localized using either a polyclonal anti-tubulin (Green and Goldman, 1983) or monoclonal antibodies directed against beta-tubulin (a gift of Dr Lester Binder, Northwestern University; Wang et al., 1993). Kinesin localization was determined using an affinity-purified polyclonal antibody directed against a highly conserved sequence (residues 335-356; Ac-CLTAEQWKKKYEKEKKKNK-Namide) in the neck region of the motor domain of conventional kinesin (Vale and Fletterick, 1997; Prahlad et al., 1998). Fluorescein- and rhodamine-labeled goat antimouse and goat anti-rabbit secondary antibodies were used in all of these studies (Jackson ImmunoRes. Lab. Inc., West Grove, PA, USA).

Membranous vesicles in extruded axoplasm were stained using the lipophilic dyes DiI, DiOC6, Neutral Red and Acridine Orange (Molecular Probes Inc., Eugene, OR, USA) (Spector et al., 1997) added to Buffer X at concentrations recommended by the manufacturer. To determine whether there was a relationship between membranous components and NIF protein, preparations of axoplasm were incubated in one of the lipophilic dyes, fixed as described above, stained with antibodies against NIF proteins, and examined by confocal microscopy.

AVEC-DIC studies on microtubule-dependent motility
In order to observe microtubule-dependent motility, axoplasm was extruded on locator coverslips (Belco Glass Inc., Vineland, NJ, USA) into approx. 50 μl of Buffer X (Brady et al., 1993). The coverslips were mounted on glass slides, sealed along their perimeters using VALAP (Kuznetsov et al., 1992; Molyneaux and Langford, 1997) leaving a space for two small wells at opposite sides of the coverslip. These preparations were then observed by video-enhanced differential interference microscopy (AVEC-DIC) using a Zeiss Axiomat equipped with a 100x objective (1.3 NA), a matched condensor (1.3 NA), and a zoom magnification of 2.5x (Kuznetsov et al., 1992; Molyneaux and Langford, 1997). To observe microtubule-dependent motility, we selected a 25x25 μm² field at the peripheral region of the axoplasmic cylinder where individual microtubules could be resolved. The positions of the microscope fields of view were determined by their relationship with the etched marking on the coverslips. Motile activity in these fields was recorded on Super VHS videotape in real time for 5 minutes. The preparation was then fixed by perfusion with 1% glutaraldehyde in Buffer X while we continued to record any activity within the field. In order to achieve a rapid perfusion of fixative, pieces of filter paper were applied to one well, while fixative was dripped through a fine bore needle into the well at the opposite end. In this fashion, the preparations of axoplasm were fixed within 30 seconds following the addition of the glutaraldehyde solution to a well. 5 minutes following the addition of fixative, the VALAP was removed and the coverslips were gently floated off the surface of the slide by using excess Buffer X. These preparations were then processed for double-label immunofluorescence using anti-NIF and anti-tubulin as described above. The positions of the particles were carefully monitored at each step of the fixation and staining procedures. The stained preparations were then observed by confocal microscopy.

The movements of the AVEC-DIC resolvable particles were analyzed using METAMORPH image analysis software. For this purpose, the images recorded on videotape were first digitized to 512x512 dpi using ADOBE PREMIER and a MIRODEC video capture board. The distances moved by the NIF particles were measured using the MEASURE DISTANCE function of METAMORPH (Prahlad et al., 1998).

RESULTS
Particulate structures containing NIF protein are present within the squid axon and in extruded axoplasm
We began our studies by determining whether structures containing NIF protein similar to the motile non-filamentous IF precursors of fibroblasts (Prahlad et al., 1998) were present in squid axons and extruded axoplasm. To this end, we stained pieces of dissected squid axon and extruded axoplasm with the polyclonal antibodies directed against the squid NIF proteins, NIF 220 and NIF 60/70, and the monoclonal antibody SMI 31 (see Materials and Methods). All three antibodies yielded indistinguishable patterns, both within the axon and in the extruded axoplasm. In addition, double-label immunofluorescence using combinations of SMI 31 and anti-NIF 220, or SMI 31 and anti-NIF 60/70, demonstrated that the same structures were recognized by each of these antibodies (>96% colocalization of anti-NIF 200 and NIF-60/70 with SMI 31, n=400 for each of the double labeled preparations; not shown).

The three NIF proteins form large numbers of neuronal IF, which appeared as a dense filamentous network in squid axons (Fig. 1a). In addition, a few punctuate structures (Fig. 1a), similar to the non-filamentous IF precursors of fibroblasts (Prahlad et al., 1998), could be detected with each of the squid NIF antibodies. These structures will be referred to as NIF particles. The NIF particles could be better visualized in preparations of extruded axoplasm. Following extrusion, the majority of the larger cytoskeletal components, including microtubules (Allen et al., 1985; Vale et al., 1985; Weiss et al., 1991) and filamentous NIF are retained in the bulk axoplasm. However, it has been shown that some microtubules, as well as numerous organelles involved in microtubule-based motility, become dissociated from the bulk axoplasm and can be detected in the peripheral regions of extruded axoplasm (Brady et al., 1982; Allen et al., 1985; Vale et al., 1985; Kuznetsov et al., 1992; Molyneaux and Langford,
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1997). Immunofluorescence observations on preparations of extruded axoplasm revealed that these regions also contained numerous NIF particles and a few short NIF (Fig. 1b). These results indicate that NIF particles, similar in morphology to the motile IF precursors (vimentin particles) in fibroblasts, are present both within the squid axon and in extruded axoplasm.

Furthermore, double-label immunofluorescence revealed that the particles contained both the SMI 31 antigen and either the NIF 220 or NIF 60/70 proteins (not shown).

**NIF particles are associated with microtubules in preparations of extruded axoplasm**

We next determined whether the NIF particles were associated with axonal microtubules. To do this, preparations of extruded axoplasm were fixed and double-labeled with each of the NIF and tubulin antibodies and examined by confocal microscopy. The distribution of NIF particles in regions of axoplasm containing microtubules revealed that the majority (approx. 68%, n=300) of NIF particles within these regions were closely associated with axonal microtubules (Fig. 2). In addition, we observed that some of the short filaments were also associated with microtubules.

**Rapid movements of NIF particles occur along axonal microtubules**

Since NIF particles were associated with microtubules, we attempted to determine whether these particles moved along microtubules at the fast rate of axonal transport. In order to do this, we first tried to visualize their motility in preparations of axoplasm. In previous studies, video-enhanced differential interference contrast (AVEC-DIC) microscopy has been used extensively for visualizing the movements of membrane-bound organelles along individual microtubules in the peripheral regions of extruded axoplasm (Brady et al., 1982; Allen et al., 1985; Vale et al., 1985; Kuznetsov et al., 1992). AVEC-DIC is capable of detecting single microtubules, and cytoplasmic particles or organelles as small as 25-50 nm (Brady et al., 1993). We reasoned, therefore, that we might be able to use AVEC-DIC to visualize the movements of some of the larger and denser NIF particles along microtubules.

![Fig. 1](image1.png)

**(a)** Confocal micrograph of a section of a fixed, permeabilized squid axon mounted onto a glass coverslip, stained with anti-NIF220. Note the presence of filamentous NIF and NIF particles (arrowheads). (b) NIF particles in the peripheral region of extruded axoplasm (arrows). Bars, 10 μm.

![Fig. 2](image2.png)

**NIF particles are associated with microtubules in the peripheral region of axoplasm.** Confocal micrographs of a microscope field showing (a) NIF particles, (b) axonal microtubules and (c) overlay of a and b. Yellow indicates the many regions of overlap between NIF particles and microtubules. Bar, 10 μm.
Numerous particles were detectable by AVEC-DIC in extruded axoplasm. The NIF particles amongst these could only be identified after the axoplasm was fixed and immunolabeled with NIF antibodies. Therefore, in order to determine whether any motile particles contained NIF proteins, the movements of all particles in a microscope field were first recorded on videotape and the axoplasm was subsequently processed for immunofluorescence. Antibodies against NIF and tubulin were used and NIF particles that corresponded to DIC particles were then identified by comparing the AVEC-DIC and the fluorescent images. The alignment and registration of the AVEC-DIC and immunofluorescence images was possible due to the preservation of the relative configurations of the microtubules as well as the markings etched onto the locator coverslips. To achieve the best possible fidelity in the alignment of these images, we monitored the axoplasmic preparations during fixation. Thus any changes in positions of the microtubules or associated particles that might have occurred during the process of fixation were also recorded in the final AVEC-DIC images with which the immunofluorescence confocal images were compared.

A comparison of the AVEC-DIC and immunofluorescence images obtained from two separate preparations of axoplasm revealed that ten of a total of 32 fluorescent NIF particles coincided in position with ten of the 52 AVEC-DIC particles. Not all the NIF particles were visible by AVEC-DIC, and we observed a slight lateral shift in the relative positions of particles and microtubules in the immunofluorescence images with respect to the AVEC-DIC images due, most likely, to differences in the optical characteristics of the two images and the various steps involved in processing the specimen. Therefore, in order to assure ourselves that the NIF particles identified by this method were not the result of a fortuitous coincidence in the positions of AVEC-DIC and fluorescent particles, the probability of such a coincidence was calculated. Using the total number of AVEC-DIC resolved particles in unfixed preparations \((n=52)\), the total number of NIF particles in the fixed/stained images captured from the same microscope field \((n=32)\), the relative areas of the images \((\text{approx.} 500 \text{ square pixels})\) and the mean area of the particles \((\text{approx.} 8 \text{ square pixels})\), we estimated such a probability to be only \(1 \text{ in } 10,000\). This is several orders of magnitude less than the observed \(1 \text{ in } 5\) AVEC-DIC particles that coincided with the fluorescent particles. We are therefore confident that we can identify a subpopulation of NIF particles using AVEC-DIC in the preparations of axoplasm, within the limits of resolution imposed by these techniques.

As mentioned above, numerous NIF particles and short NIF filaments associated with MT were visible following the fixation.
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and staining of the extruded axoplasm. Our studies were restricted to behavior of the NIF particles. Ten NIF particles visible by AVEC-DIC were analyzed using the previously recorded videotapes. Of the ten NIF particles, eight were observed to move along axonal microtubules (for an example, see Fig. 3). The remaining two particles were not associated with microtubules and remained stationary for the duration of our observations. The movements of the eight NIF particles were discontinuous or saltatory (Rebhun, 1967) in nature. These motile NIF particles could be followed for total distances of approx. 5-7 μm along microtubules. The instantaneous rates of movement calculated for these distances ranged between 0.5-1.0 μm/second. In addition, each particle moved predominantly in one direction along a given microtubule. These observations indicate that NIF particles are capable of moving at the rate of fast axonal transport along microtubules.

**NIF particles are associated with kinesin and are not membrane-bound**

We then examined whether kinesin, the microtubule-dependent motor known to be involved in fast axonal transport (Vale et al., 1985; Hirokawa, 1997; Gindhart et al., 1998), was associated with the particles. In order to do this, an antibody raised against a peptide whose sequence was derived from the neck region of the motor domain of human ubiquitous kinesin was used (Vale and Fletterick, 1997; also see Prahlad et al., 1998). This sequence is highly conserved among kinesins and is 80% identical to a sequence within the motor region of squid kinesin heavy chain (Fig. 4a; Kosik et al., 1990). In a western blot of extruded axoplasm the antibody reacted with a major band of the appropriate molecular mass of 120 kDa (Fig. 4b). Eight axoplasmic preparations were then fixed and double-labeled with anti-NIF and anti-kinesin. Approximately 70% (n=400) of the NIF particles visualized in these preparations colocalized with kinesin (Fig. 5a-c). These observations suggest that a member of the kinesin family of motor proteins is responsible for the rapid microtubule-dependent transport of the NIF particles. In addition, some filamentous NIF also stained with kinesin antibody. These observations suggest that kinesin may also be involved in the fast transport of short NIF (see Fig. 5a-c).

Since kinesin has been shown to be mainly responsible for the movement of membranous organelles along microtubules, we examined whether the NIF particles were membrane-bound. In five preparations of axoplasm fixed and double-stained with a lipophilic dye (Spector et al., 1997; see Materials and Methods) and NIF antibodies, we observed that <1% (n=400) of the NIF particles showed an obvious association with membranes (Fig. 5d-f), suggesting that NIF particles were not membrane-bound. These studies suggest that kinesin is involved in the transport of non-membrane-bound protein particles in axons.

**DISCUSSION**

Recent observations on the motility of GFP-labeled
neurofilaments in live mammalian nerve cells (Wang et al., 2000; also see Lasek et al., 1993) have shown that neurofilaments up to 15.8 μm long can be translocated along axons at rates up to 0.89 μm/second, but pause frequently. This result is in contrast to previous reports that cytoskeletal proteins are transported at slow rates (0.001-0.05 μm/second) in axons. Taken together with the results described in this study it is now possible to resolve the rapid movements of individual transported complexes of neurofilament protein and, thus, begin to dissect the mechanisms underlying their rapid movements. Our studies suggest that nonfilamentous neurofilament protein (NIF) particles can also move at fast rates of axonal transport. This fast transport of NIF particles, and possibly short NIF, occurs along individual axonal microtubules. Immunofluorescence labeling studies with kinesin antibodies further suggest that at least one of the motors involved in these microtubule-dependent movements is a member of the kinesin family of proteins. In addition, since NIF particles do not stain with lipophilic dyes, they appear distinct from the previously described membranous organelles that move rapidly along microtubules in squid axoplasm (Brady et al., 1982).

It has been shown that the subcellular organization of fully polymerized NIF is dependent on microtubules and their associated proteins (Dahl et al., 1980; Llorens and Dememes, 1996). The transport of NIF proteins along axonal microtubules and their colocalization with kinesin as described in this study suggests a mechanism to explain why this might be the case. This dependence of polymerized IF networks on microtubules and kinesin is also apparent in the case of non-neuronal cells such as fibroblasts (Prahlad et al., 1998; Gyoeva and Gelfand, 1991; Goldman, 1971), suggesting that the mechanisms underlying the assembly and regulation of cytoskeletal architecture are similar in neuronal and non-neuronal cells.

It is not clear whether the movements of the NIF particles along microtubules occurs in an anterograde or retrograde direction. However, the association of kinesin with the majority of NIF particles, as determined by the use of antibodies directed against ubiquitous conventional kinesin, suggests that at least a significant portion of NIF particles move in an anterograde direction. The immunolocalization studies using anti-tubulin and anti-NIF have also allowed us to determine that NIF protein is probably moved using the microtubule-based (Brady et al., 1982), rather than the microfilament-based (Kuznetsov et al., 1992), transport mechanism present in axons. This is supported by the finding that kinesin is associated with large numbers of NIF particles and some short NIF filaments. It is possible, however, that neurofilament proteins are associated with more than one type of motor. In support of this, a member of the kinesin family of proteins has also been previously implicated in the slow movement of neurofilament protein in growing axons (Yabe et al., 1999; Shea et al., 1997), and both kinesin and dynein associate with the radial spoke components transported along microtubules within the Chlamydomonas flagellum (Cole et al., 1998; Pazour et al., 1998).

The observations made in this study have some bearing on the nature of NIF proteins involved in axonal transport (Bass and Brown, 1997; Hirokawa et al., 1997). In particular, it remains controversial whether cytoskeletal proteins are transported in the form of polymers or smaller subunits (Bass and Brown, 1997; Hirokawa et al., 1997). This controversy has been partially resolved by the demonstration that short filaments comprising NIF proteins can move as a fast-transported component within axons (Wang et al., 2000). Our studies suggest that a nonfilamentous form of NIF, NIF particles, can also be transported at the fast rate of axonal transport (also see Terada et al., 1996). The short NIF and the NIF particles are also highly reminiscent of two of the most prominent assembly states of vimentin that have been visualized in fibroblasts in vivo (Prahlad et al., 1998; Yoon et al., 1998). The morphology of the fast-moving NIF particles reported here and their similarity to vimentin particles in fibroblasts (Prahlad et al., 1998) suggests that they consist of nonfilamentous oligomers of NIF proteins (also see Terada et al., 1996). The short NIF (see Wang et al., 2000), on the other hand, appear morphologically similar to microtubule-dependent, slower moving vimentin ‘squiggles’, which are a result of the regional assembly of vimentin particles into short IF (Prahlad et al., 1998). Thus, as appears to be the case in fibroblasts (Yoon et al., 1998; Prahlad et al., 1998), microtubule-dependent transport could be involved in the movement of both nonfilamentous and filamentous protein along axons. It is possible therefore that NIF protein is transported in these different assembly states due to the varying regional requirements of the neuron (Nixon and Longvinenko, 1986). It is also worthwhile noting that there is growing evidence that protein synthesis can occur throughout the axon (Alvarez et al., 2000). If this is the case, the NIF particles and short filaments might represent complexes of newly transcribed protein that are being shuttled to their final destinations within axons while in different states of the NIF assembly process.

It is unclear at the present time whether there is a difference between the distance traversed, and net rate of transport of the NIF particles reported here and the short NIF reported in mammalian neurons (Wang et al., 2000). Our sample size of eight NIF particles is insufficient to determine whether this is the case, and future studies are required to resolve this issue. In the extruded axoplasm system, it was impossible to distinguish between membranous organelles and NIF particles by the nature of their motility along axonal microtubules. Thus, although the peak rates of transport of both these structures correspond to that of fast transport, it is conceivable that some NIF particles might engage in fast transport more frequently, and for longer distances, in ways similar to the membrane-bound vesicles (Brady et al., 1982; Pollock et al., 1999).

Although our studies have focused on NIF, similar mechanisms of fast transport could be involved in the distribution of other cytoskeletal proteins. In support of this, there is evidence that varicosities which stain with antibodies directed against NIF proteins and/or tubulin (Hollenbeck and Bray, 1987) and actin (Koenig et al., 1985), move within axons at fast rates. Furthermore, nonfilamentous complexes containing NIF protein, tubulin and spectrin have been purified from brain tubulin preparations (Weisenberg et al., 1985). Preliminary observations of squid axoplasm indicate that a subset of the NIF particles stain with tubulin antibodies (our unpublished results), suggesting that they might correspond to the rapidly transported varicosities seen in live neurons (Hollenbeck and Bray, 1987). In the future, it will be necessary to purify and characterize the various forms of neurofilament...
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Protein from nerve tissue in order to determine the relationships between their structure, organizational states, motile properties and associated motor proteins.

In summary, the results of this study suggest that particles containing NIF proteins in squid axoplasm can move on microtubules at fast transport rates. These particles are not membrane-bound, and are associated with kinesin. Although the significance of the microtubule-dependent fast transport of NIF particles is not known, it has been shown that the advance of growth cones in developing neurons requires fast axonal transport and cytoskeletal assembly (Martensen et al., 1993). The microtubule-dependent fast delivery of NIF proteins could provide neurons with the capacity to more precisely target and deliver subunits regionally throughout their cytoplasm. The local assembly of these subunits into polymerized NIF could help to explain the regional variations in NIF numbers reported along the lengths of axons (Nixon and Longyinvenko, 1986).

Fast microtubule-dependent axonal transport of cytoskeletal proteins might also be important for the maintenance and turnover of cytoskeletal elements located in the most distal regions of axons of extraordinary length. Furthermore, alterations in the fast transport of NIF particles due to defects in microtubule-dependent transport mechanisms could be responsible, in part, for the abnormal accumulations of NIF that typify Parkinson’s disease, amyotrophic lateral sclerosis and Giant Axonal Neuropathy (Sim et al., 1978; Goldman et al., 1983; Bousquet et al., 1996).

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