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Phospho-Dependent Association of Neurofilament Proteins With Kinesin In Situ

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Recent studies demonstrate co-localization of kinesin with neurofilament (NF) subunits in culture and suggest that kinesin participates in NF subunit distribution. We sought to determine whether kinesin was also associated with NF subunits in situ. Axonal transport of NF subunits in mouse optic nerve was perturbed by the microtubule (MT)-depolymerizing drug vinblastine, indicating that NF transport was dependent upon MT dynamics. Kinesin co-precipitated during immunoprecipitation of NF subunits from optic nerve. The association of NFs and kinesin was regulated by NF phosphorylation, since (1) NF subunits bearing developmentally delayed phospho-epitopes did not co-purify in a microtubule motor preparation from CNS while less phosphorylated forms did; (2) subunits bearing these phospho-epitopes were selectively not co-precipitated with kinesin; and (3) phosphorylation under cell-free conditions diminished the association of NF subunits with kinesin. The nature and extent of this association was further examined by intravitreal injection of ^{35}S-methionine and monitoring NF subunit transport along optic axons. As previously described by several laboratories, the wave of NF subunits underwent a progressive broadening during continued transport. The front, but not the trail, of this broadening wave of NF subunits was co-precipitated with kinesin, indicating that (1) the fastest-moving NFs were associated with kinesin, and (2) that dissociation from kinesin may foster trailing of NF subunits during continued transport. These data suggest that kinesin participates in NF axonal transport either by directly translocating NFs and/or by linking NFs to transporting MTs. Both Triton-soluble as well as cytoskeleton-associated NF subunits were co-precipitated with kinesin; these data are considered in terms of the form(s) in which NF subunits undergo axonal transport. Cell Motil. Cytoskeleton 45:249–262, 2000. © 2000 Wiley-Liss, Inc.

Key words: neurofilaments; axonal transport; kinesin; motor proteins; phosphorylation; cytoskeleton

INTRODUCTION

Like all constituents of the axonal cytoskeleton, NFs are synthesized exclusively within the neuronal perikaryon and are subsequently delivered to the axon by a process referred to as axonal transport [Hirokawa, 1993; Nixon, 1993, 1998a, b]. While the mode of NF axonal transport has not been determined, compelling evidence has been presented that phosphorylation regulates this process [deWaegh et al., 1992; Hoffman et al., 1985; Jung and Shea, 1999; Komiya et al., 1986; Lewis and Nixon, 1988; Nixon et al., 1994; Shea et al., 1993; Watson et al., 1993; Zhang et al., 1997]. NFs are among the most highly phosphorylated proteins within axons. Distinct kinases phosphorylate the N-terminal and C-terminal portion of NFs [Julien et al., 1983; Pant et al., 1979; Runge et al., 1981; Schekert and Lasek, 1982;

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Sihag and Nixon, 1989, 1990, 1991] and, in doing so, are thought to modulate aspects of NF assembly and interaction with other cytoskeletal proteins. Site-specific NF phosphorylation would also be likely to regulate the association of NFs with their putative transport motor system [e.g., Baas and Brown, 1997; Jung and Shea, 1999; Lasek et al., 1992, 1993; Nixon, 1993, 1998a, b].

Axonal transport is divided into two overall categories based on relative speed. These include so-called "fast axonal transport," which includes membranous organelles, and "slow axonal transport," which includes those proteins that comprise axonal cytoskeletal polymers, including NFs, microtubules (MTs), and their associated proteins. While motor proteins that mediate fast axonal transport have been described, motor(s) responsible for NF axonal transport remain undisclosed [e.g., see Hirokawa, 1997].

Recent studies have demonstrated intracellular associations between the microtubule motor kinesin and NF subunits in cultured neurons and neuroblastoma [Yabe et al., 1999]. Real-time video microscopy of transfected cells revealed that oligomeric assemblies containing kinesin and NF subunits underwent axonal transport in these cells at a rate consistent with slow axonal transport [Yabe et al., 1999]. NF subunits also co-purified with a standard microtubule motor preparation generated from mouse brain, suggesting that NF subunits and kinesin may be associated in situ [Yabe et al., 1999]. Should kinesin be associated with NFs in situ, we reasoned that kinesin and NFs should co-precipitate under certain conditions. We present data herein indicating that, as in cultured neurons, kinesin is associated with NFs in optic axons in situ and that this association is disrupted by NF phosphorylation. We also demonstrate that NF axonal transport is dependent upon an intact MT system, and that kinesin is selectively associated with the front-most portion of the transporting wave of newly-synthesized NF subunits within axons in situ. The implications of these latter phenomena for a role for kinesin in NF axonal transport is discussed.

MATERIALS AND METHODS

Injection of Radiolabel and Harvesting of Tissues

Murine retinal ganglion cells were radiolabeled in situ by injection of 70 μCi ^{35}S-methionine in a total volume of 0.2 μL via a pulled glass capillary pipette into the vitreous of anesthetized mice [Shea et al., 1997]. Mice were sacrificed by cervical dislocation at intervals of 12 h to 14 days following injection. Retinas were dissected away from the rest of the eye and optic axons dissected into 9 × 1.1 mm segments on a glass slide on dry ice. Retinas and segments from 5–11 mice were pooled and homogenized in 1% Triton X-100 in 50 mM Tris (pH 6.9) containing 2 mM EDTA, 1 mM PMSF and 50 μg/ml leupeptin at 4°C by 50 strokes in a tight-fitting glass-Teflon homogenizer [Nixon and Logvinenko, 1986; Shea et al., 1997]. The Triton-insoluble cytoskeleton was sedimented by centrifugation 15,000*g* for 15 min as described [Chiu and Norton, 1982].

Fractionation and Enrichment of Kinesin

Kinesin was enriched from mouse brain via a standard microtubule motor protein preparation [Saxton, 1994] as described [Yabe et al., 1999]. Mouse brains (25 brains, approximately 10 g total weight) were homogenized at 4°C with a glass-teflon homogenizer in buffer A [0.1M PIPES (pH 6.9) containing 0.9M glycerol, 5 mM EGTA, 2.5 $MgSO_4$, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μM phenylmethylsulfonyl fluoride (PMSF), and 0.1mg/ml soybean trypsin inhibitor]. The homogenate was centrifuged at 1000*g* at 4°C for 15 min to sediment cellular debris ("P1") and the resulting supernatant ("S1") was warmed to 25°C. The following were added to the warmed supernatant: 1 mM GTP and 20 μM taxol followed by incubation at 37°C for 20 min (to induce MT polymerization), then 50 μM AMP-PNP, 10 mM glucose, and 2 U/ml hexokinase, followed by incubation at 25°C for 20 min (to induce binding of motor proteins). The supernatant was then centrifuged at 100,000*g* at 25°C for 60 min to sediment MTs and associated motor proteins. The resulting pellet ("P2") was resuspended in buffer A containing 2 mM GTP, 20 μM taxol, 10 mM ATP, 10 mM $MgSO_4$, and 10 mM ATP (to foster selective release of motor proteins from assembled MTs), the pellet ("P3") was discarded. To deplete any residual tubulin from the resulting supernatant ("S2"), an additional 1 mM GTP and 20 μM taxol were added to the resulting supernatant, and the above centrifugation repeated [Saxton, 1994].

As described [Yabe et al., 1999], CNS contains numerous NFs and NF oligomers that sediment following centrifugation at 100,000*g* for 1 h, as well as monomers/small oligomers that are not sedimented [Jung et al., 1998; Shea et al., 1990, 1997]. However, since this preparation alternately utilizes pellet and supernatant fractions resulting from centrifugation steps [Saxton, 1994], it eliminates artifactual contamination with NF subunits. High-speed sedimentable NF polymers would indeed initially sediment along with MTs following the first high-speed centrifugation in this motor protein preparation; however, they would be depleted by the subsequent centrifugations (e.g., see P3 in Fig. 3) utilized to clarify the supernatant containing released motor proteins. Similarly, NFs monomers or small oligomers not sedimentable by high-speed centrifugation [Jung et al., 1998; Shea et al., 1990, 1997] would be discarded during

subsequent steps in which only sedimented material is retained (e.g., see S2 in Fig. 3).

Purification of NF Subunits

Bovine spinal cords were homogenized in 100 mM PIPES (pH 6.6) containing 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT, and 1 μg/ml leupeptin. NFs were recovered in the void volume following Sepharose CL-4B gel chromatography [Hisanaga and Hirokawa, 1988; Takeda et al., 1994]. As described [Takeda et al., 1994], a single assembly/disassembly cycle was sufficient to eliminate tubulin and other contaminating proteins. Isolated NFs were disassembled in urea buffer [100 mM phosphate buffer (pH7.5) containing 6M urea, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5M PMSF, and 1 μg/ml leupeptin], and subjected to DE-52 chromatography in urea buffer. NF subunits were eluted over a range of 0.15M–0.2M NaCl. Subunits were dialyzed overnight at 4°C 20mM PIPES (pH6.6) containing 1 mM EGTA, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.17 NaCl [e.g., Takeda et al., 1994] to remove urea. As expected, glial fibrillary acidic protein and vimentin co-purified with NFs in void volume fractions from gel filtration; these intermediate filament species were eluted in fractions distinct from that of NF subunits in ion-exchange chromatography as confirmed by immunoblot analysis (not shown).

Cell-Free Phosphorylation

Aliquots (50 μL) of the above-mentioned MT motor protein preparation were incubated for 4 h at 37°C with constitutively active MAP kinase (50 μg; UBI, Lake Placid, NY) in 50 mM Tris-HCl (pH 7.4) containing 5 mM EDTA, 5 mM $MgCl_2$, 100 μM ATP, and 1 mM DTT as described [Veeranna et al., 1998].

Gel Electrophoresis, Autoradiography, and Immunoblot Analyses

NF subunits and kinesin were, respectively, immunoprecipitated from various CNS homogenates and the above fractions using 1:150 dilutions of a polyclonal antibody that quantitatively immunoprecipitates all 3 NF subunits (R39) [Shea et al., 1997] or a pan-specific polyclonal anti-kinesin antibody raised against bovine brain kinesin (Cytoskeleton, Inc., Denver, CO), followed by protein A-sepharose (10 mg; Sigma, St. Louis, MO) as described [Shea et al., 1990, Shea et al., 1997]. The anti-kinesin antibody utilized herein has been previously shown not to immunoprecipitate purified NF subunits in the absence of kinesin [Yabe et al., 1999]. In some experiments, the post-precipitate generated following immunoprecipitation with anti-kinesin was subsequently immunoprecipitated with R39. Immunoprecipitated material, along with the above homogenates and fractions, was subjected to SDS-gel electrophoresis on linear 7% acrylamide gels. Gels were either Coomassie-stained, photographed, and, in the case of radiolabeled optic pathway, dried and placed against Kodak X-Omat film to generate autoradiographs, or the separated proteins were transferred to nitrocellulose. Nitrocellulose replicas were probed as described [Shea et al., 1997] with 1:100–1:1,000 dilutions of R39, monoclonal antibodies directed against phosphorylated (SMI-31) or non-phosphorylated (SMI-32) NF epitopes common to NF-H and NF-M (Sternberger Monoclonals, Inc., Jarretsville, MD), polyclonal antibodies (L3 and M2) generated in this laboratory against purified NF-L and NF-M, respectively [Jung et al., 1998; Jung and Shea, 1999], a polyclonal antibody (F34) directed against fodrin [Sihag et al., 1996], a monoclonal antibody (RT97) directed against a developmentally-delayed, C-terminal phospho-epitope of NF-H [Anderton et al., 1982; Dahl et al., 1988, 1992], a monoclonal antibody (JJ8) directed against a developmentally-delayed phospho-epitope common to NF-H and NF-M [Dahl et al., 1988, 1992], or with the above anti-kinesin antibody. As in our previous studies, pre-immune sera, commercially-obtained non-immune sera, or protein A-Sepharose alone did not precipitate any NF- or kinesin-related polypeptides, and omission of primary antibodies in immunoblot analysis failed to generate reactive species.

Densitometric Analyses and Calculation of Transport Rates

Gels, autoradiographs, and nitrocellulose replicas were digitized via a UMax scanner equipped with a transparency adaptor operated by a Macintosh Power PC. Densitometric analyses of digitized images were carried out via NIH Image software by encircling the entire band with the program's freehand selection tool as described previously [Cressman and Shea, 1995]. Since the NF triplet co-migrated along optic axons, we subsequently present densitometric data only for NF-L for simplicity only [e.g., Lasek et al., 1993]. Densitometric calculations using the NF triplet yielded identical relative distributions (not shown). The transport rate of NF subunits, expressed as mm/day, was determined for the "50th percentile" of radioactivity essentially as described previously [Hoffman et al., 1983] with minor modifications [Jung et al., 1998]. The location of the 50th percentile, herein referred to as the "," at various times following injection of radiolabel was operationally defined as that segment into which ≥50% of radiolabeled subunits had entered or traversed [Jung et al., 1998].

Total mean density (density per total area of immunoreactive band) was calculated for autoradiographs. To facilitate comparison of distribution of radiolabeled subunits at different post-injection intervals, total subunit

radiolabeled recovered from the retina and all axonal segments was defined as 100% for each respective time interval. The relative amount of radiolabel in each segment was then expressed as a percentage of the total at that interval. The distribution of tubulin and fodrin was quantified in an identical manner. Incorporation of radiolabeled tubulin into Triton-insoluble structures [Tomoko and Komiya, 1989] in the presence and absence of 0–500 μM vinblastine was calculated by dividing the mean densitometric intensity of insoluble tubulin in retinas by the sum of insoluble and soluble retinal tubulin under each condition. All such comparisons were performed for individual autoradiographs and immunoblots, and resultant respective values were then pooled. All tables and graphs of densitometric analyses represent averages derived from at least two independent experiments. Each sample in radiolabeling analyses was generated from pools of both optic pathways from 4–11 mice. Multiple autoradiographs of differing exposures were generated to insure linearity.

RESULTS

NF Transport In Situ Is Dependent Upon MTs

Axonal transport of radiolabeled NF subunits was monitored from 12 h to 14 days post injection of ^{35}S-methionine radiolabel by autoradiographic analyses of NF subunits immunoprecipitated with a polyclonal antibody (R39) that quantitatively immunoprecipitates all 3 NF subunits regardless of phosphorylation state (Fig. 1) [see also Shea et al., 1997; Jung et al., 1998]. Consistent with previous studies [e.g., Watson et al., 1989; Nixon and Logvinenko, 1986; Lasek et al., 1992, 1993; Jung et al., 1998], transport of NF subunits into optic axons initiated within hours after injection of radiolabel, and the profile of radiolabeled subunits broadened during continued transport (Fig. 1). Intravitreal injection of the MT-depolymerizing drug vinblastine along with radiolabel induced a dose-dependent inhibition of incorporation of newly synthesized tubulin into Triton-insoluble structures, which provided an index of decreased formation of stable MTs [e.g., see Tomoko and Komiya, 1989]. Vinblastine also induced a dose-dependent inhibition of axonal transport of tubulin and NFs (Fig. 2). Inhibition of NF axonal transport by vinblastine was not a reflection of an overall inhibition of axonal transport, since, for example, the transport of cytoskeleton-associated fodrin was not inhibited (Fig. 2). The effects of vinblastine on NFs were confined to transport into and along axons rather than NF assembly or association with Triton-insoluble structures, since increasing levels of radiolabeled NFs were recovered within retinal cytoskeletons with increasing vinblastine concentrations (Fig. 2). These data indicate that NF axonal transport is dependent in some capacity upon MT dynamics.

NFs and Kinesin Co-Purify in Cell-Free Analyses

The above data is consistent with transport of NFs either by direct interaction with a MT motor, or as cargo "piggy-backing" on MTs that are themselves undergoing anterograde transport. Should NFs interact with a MT motor, NF subunits, and their motor would be expected to co-purify under certain conditions. We investigated potential interactions between NFs and the anterograde MT motor kinesin, since kinesin co-localizes with vimentin [Liao and Gundersen, 1998] and NF subunits [Yabe et al., 1999], and is involved in the distribution and transport of vimentin [Gyoeva and Gelfand, 1991; Prahlad et al., 1998] and NF subunits [Yabe et al., 1999] within cultured cells.

We have previously demonstrated that NF subunits co-purified with a standard MT motor preparation from mouse brain [Yabe et al., 1999]. Moreover, when kinesin was immunoprecipitated from this apparently tubulin-free preparation, NF subunits co-precipitated along with kinesin, confirming that NFs were, indeed, associated in some capacity with kinesin [Yabe et al., 1999].

We next examined whether kinesin associated with purified NF subunits. This could not be conclusively determined by examination of preparations of intact NFs from CNS, since some vimentin and glial-fibrillary acidic protein were retained in such preparations (not shown). Vimentin is known to bind kinesin [Liao and Gundersen, 1998] and glial fibrillary acidic protein may be expected to do so; accordingly, co-purification of kinesin with cytoskeletal preparations or even intermediate filament preparations could be attributed to these non-NF intermediate filaments. To clarify the putative association of kinesin with NFs, NFs were dissociated and subunits were purified by a series of gel filtration and ion-exchange chromatography (Fig. 3). The resulting NF subunit-containing fractions were mixed with the above motor protein preparation, and these mixtures were subjected to immunoprecipitation with anti-NF and anti-kinesin antibodies. Immunoprecipitation of kinesin with an anti-kinesin antibody also resulted in co-precipitation of NF-H and NF-M, but not NF-L, subunits (Fig. 3). Since all 3 NF subunits were present in the motor preparation itself [Yabe et al., 1999], and since purified NF-H and NF-M themselves undergo phospho-dependent interactions that can lead to co-precipitation of either subunit following immunoprecipitation of the other [Eyer and Leterrier, 1988; Leterrier et al., 1996; Shea and Beermann, 1994], we cannot firmly conclude whether kinesin associates with both NF-H and NF-M or exclusively with either subunit. It is clear, however, that kinesin preferentially interacts with NF-H or NF-M, rather than NF-L.

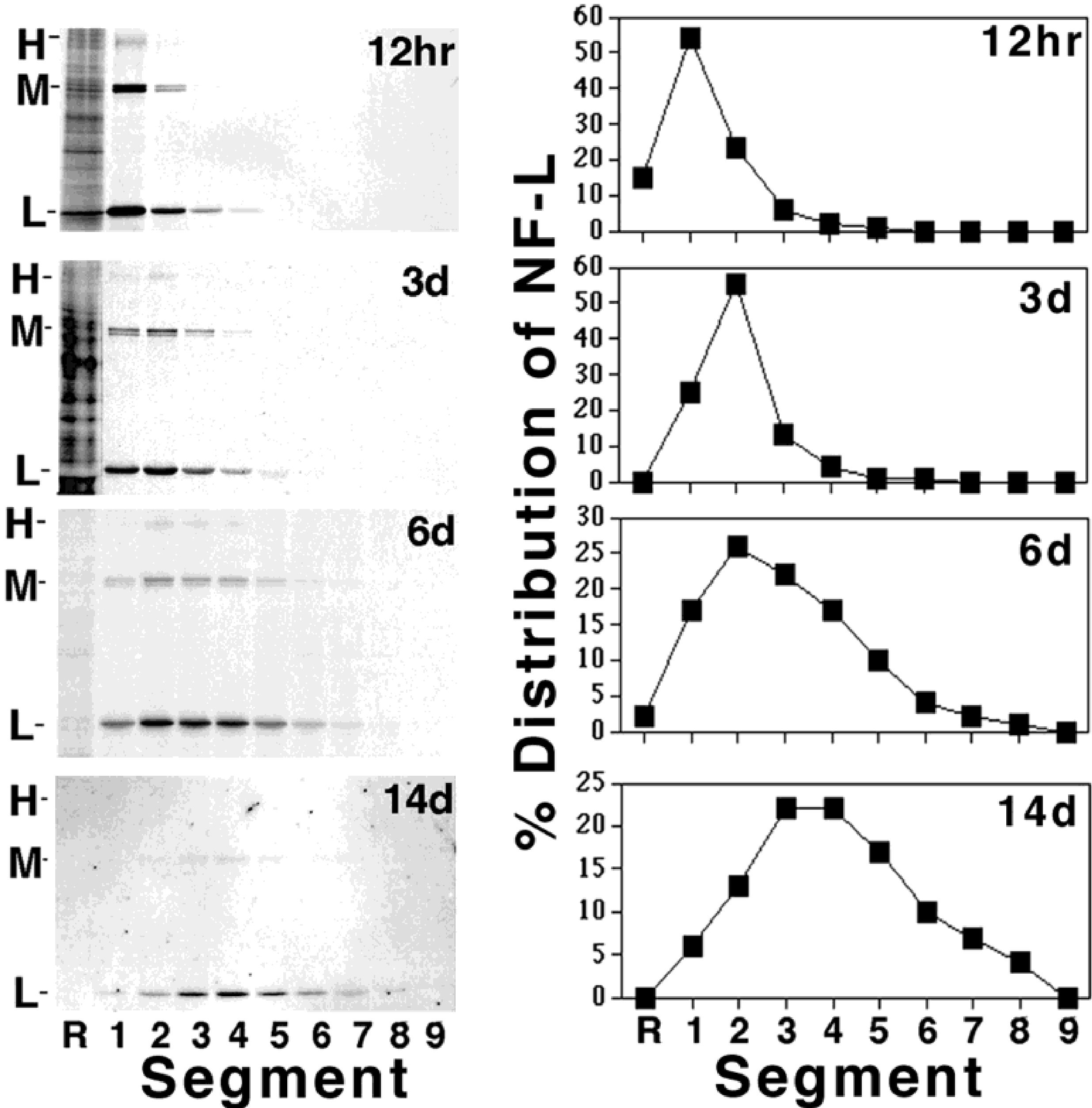

Fig. 1. Axonal transport of NFs. Autoradiographs of NFs immunoprecipitated with R39 from cytoskeletons or retinas and optic axon segments at various intervals following intravitreal injection of radiolabel. The accompanying graphs present densitometric analysis of the distribution of NF-L proteins within retinas and along the axonal length; values represent the percentage of total axonal radioactivity recovered within retinas and respective segments. Consistent with prior studies (see text), the peak of radiolabeled NF subunits undergoes a progressive broadening during continued transport.

These findings further suggest that those NF-L subunits co-sedimented within the MT motor preparation (and from optic pathway; below) represent those NF-L subunits that are assembled with either NF-H and/or NF-M, rather than monomeric NF-L subunits.

Phosphorylation Regulates the Association of NFs and Kinesin

Following attainment of their "mature" apparent molecular weight [Julien and Mushynski, 1983], the phosphorylation state of NF-H and NF-M can be further probed by

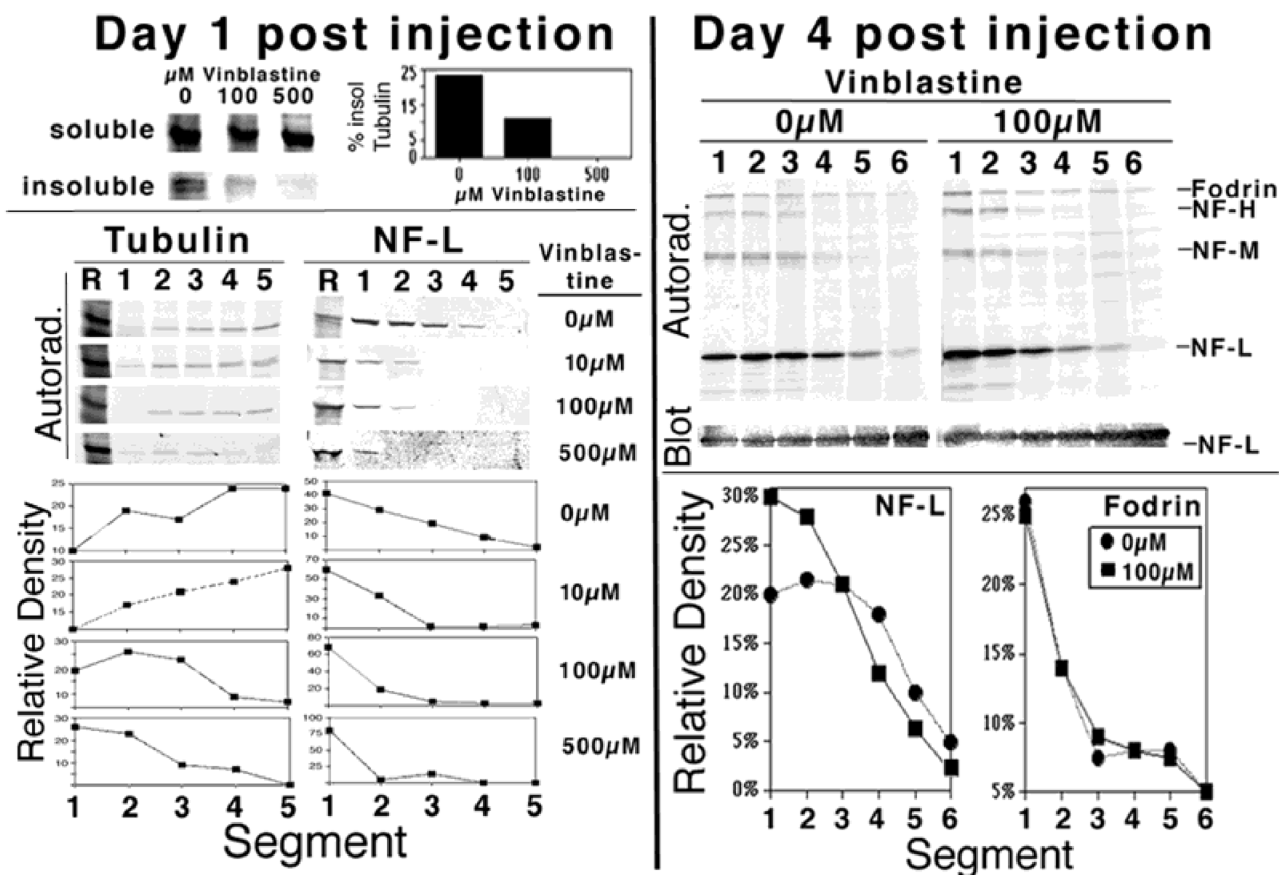

Fig. 2. NF axonal transport is dependent upon MT dynamics. Autoradiographs of tubulin immunoprecipitated from Triton-soluble and -insoluble fractions and NF-L and fodrin immunoprecipitated from Triton-insoluble cytoskeletons at days 1 and 4 after intravitreal injection of radiolabel along with various concentrations of vinblastine as indicated. *Day 1 after injection:* **Top:** Autoradiographs of tubulin in Triton-soluble and -insoluble fractions of retinas; the accompanying graph presents the % of total radiolabeled tubulin recovered within cytoskeletons. Note that vinblastine inhibits incorporation of radiolabeled tubulin into Triton-insoluble structures, indicative of a decrease in formation of stable MTs (see text). **Bottom:** Autoradiographs of Triton-soluble tubulin and Triton-insoluble NF-L within in the presence and absence of various concentrations of vinblastine. The accompanying graphs present densitometric analysis of the distribution of these proteins along the axonal length. Note inhibition of transport of tubulin and NF-L with increasing vinblastine concentrations. *Day 4 after injection:* Autoradiographic analyses of fodrin and NF subunits in axonal cytoskeletons; the accompanying graphs depict the relative distribution of fodrin and NF-L along the axonal length in the presence and absence of vinblastine. Note that vinblastine inhibits axonal transport of NF-L but not fodrin. Immunoblot analysis of NF-L from the same samples confirms that the reduction in axonal transport was not due to overall depletion of pre-existing NFs.

the presence of developmentally delayed phospho-epitopes that are recognized by well-characterized monoclonal antibodies [Anderton et al., 1982; Dahl et al., 1988; Sternberger and Sternberger, 1983]. We took advantage of these properties to ascertain the impact of NF phosphorylation on the association of NFs with kinesin in situ.

We compared the phosphorylation state of NF subunits that were retained within a standard MT motor preparation generated from brain as described [e.g., Yabe et al., 1999] vs. those that were depleted during generation of this preparation. Notably, while some 200 kDa NF-H co-purified with the motor preparation, 200 kDa NF-H bearing a developmentally-delayed C-terminal phospho-epitope was excluded from this preparation (Fig. 4). Analysis of the motor preparation with a phospho-dependent antibody (SMI-32) that recognizes NF-H only when its epitope is not phosphorylated further demonstrated the retention of faster-migrating, hypophosphorylated NF-H within the motor preparation (Fig. 4). Selective retention of certain phospho-isoforms of NF-H argues against artifactual contamination of this preparation with NFs, and instead supports that prior conclusion that NFs co-purify with this preparation due to an association with kinesin [Yabe et al., 1999].

Similarly, when kinesin was immunoprecipitated from optic pathway, which contains multiple phospho-variants of 200 kDa NF-H and NF-M [Jung et al., 1998; Lewis and Nixon, 1988; Shea et al., 1997], a portion of

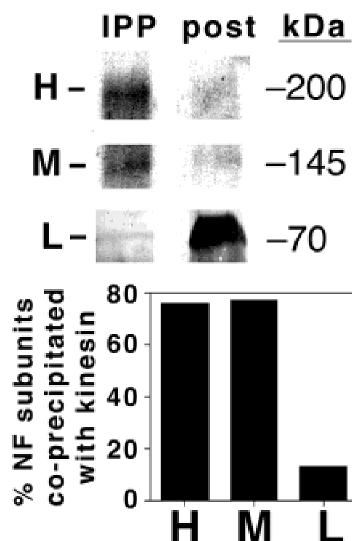

Fig. 3. Purified NF subunits co-precipitate with kinesin. NFs were purified by a series of gel filtration and ion-exchange chromatography. Aliquots of these subunits and the MT motor preparation were combined, and the mixture was subjected to immunoprecipitation with anti-kinesin followed by immunoblot analyses of immunoprecipitates and non-precipitated material with anti-NF antibodies. The relative migration of immunoreactive subunits are indicated on the right. Note that the bulk of NF-H and NF-M, but not NF-L, were co-precipitated with kinesin. The presence of both NF-H and NF-M within in the motor preparation, and the association of these two subunits via their C-terminal sidearms [Shea and Beermann, 1994], precludes determination of whether or not kinesin binds exclusively or preferentially to either of these two subunits.

the 200 kDa NF-H and 145 kDa NF-M were co-precipitated, but NFs bearing certain C-terminal phospho-epitopes were selectively not co-precipitated with kinesin (Fig. 5). These findings indicate that one or more C-terminal NF phosphorylation event interferes with the interaction of NFs with kinesin. To validate further the putative association between NFs and kinesin, we attempted to determine whether or not kinesin would co-precipitate from CNS along with immunoprecipitation of NF subunits; indeed, kinesin was co-precipitated from optic pathway homogenates with the phosphate-independent polyclonal anti-NF antibody R39 (Fig. 5).

The apparent negative regulatory influence of NF phosphorylation on their association with kinesin was further probed by phosphorylation of NF subunits within the motor preparation. To accomplish this, the motor preparation was incubated with MAP kinase, which phosphorylates C-terminal regions of NF-M [Li et al., 1999; Veeranna et al., 1998]. Immunoblot analysis confirmed MAP kinase-mediated phosphorylation of the hypophosphorylated NF subunits in our motor preparation, as indicated by decreased NF electrophoretic mobility (Fig. 6). In addition, co-precipitation along with kinesin of resultant slower-migrating isoforms was selectively reduced, while faster-migrating forms were still co-precipitated, confirming that phosphorylation regulated the association of NFs with kinesin.

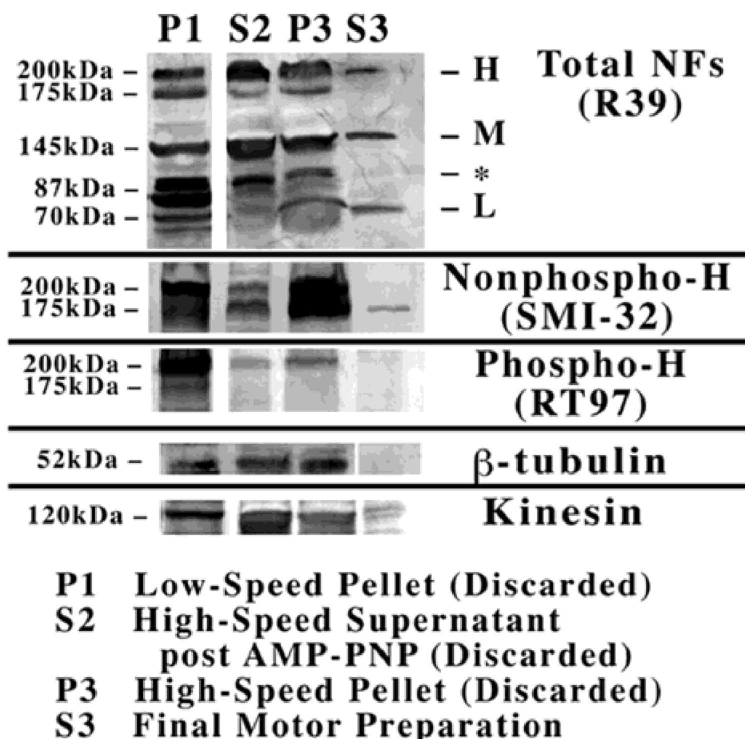

Fig. 4. Certain NF phospho-isoforms co-purify with an MT motor preparation. Immunoblot analysis of a MT motor preparation and various fractions generated during its preparation (see Materials and Methods) with an antibody (R39) directed against all NF subunits, an antibody (RT97) that recognizes a developmentally-delayed phospho-epitope of NF-H, an antibody (SMI-32) that recognizes a phosphorylation site on NF-H and NF-M only when that site is not phosphorylated, an antibody directed against β-tubulin, and the pan-specific antibody against bovine brain kinesin as indicated. Only the relevant regions of immunoblots are presented. Additional unclassified immunoreactive species ("*"), prominently labeled by R39, were not retained in the motor preparation. Note the lack of tubulin in the motor preparation. Note further that, while the non-restrictive antibody R39 demonstrates the presence of 200 kDa NF-H within the motor preparation, RT97-immunoreactive NF-H is not retained within this preparation. Moreover, SMI-32 demonstrates the presence of less phosphorylated NF-H variants within the motor preparation. Fractions that were discarded during generation of this preparation (P1, S2, and P3, see Materials and Methods) are included for comparative purposes and positive controls for antibody reactivity. All panels are derived from multiple gels from the same preparation. All lanes are from the same immunoblot and were processed simultaneously; some lanes were subsequently separated for presentation purposes only.

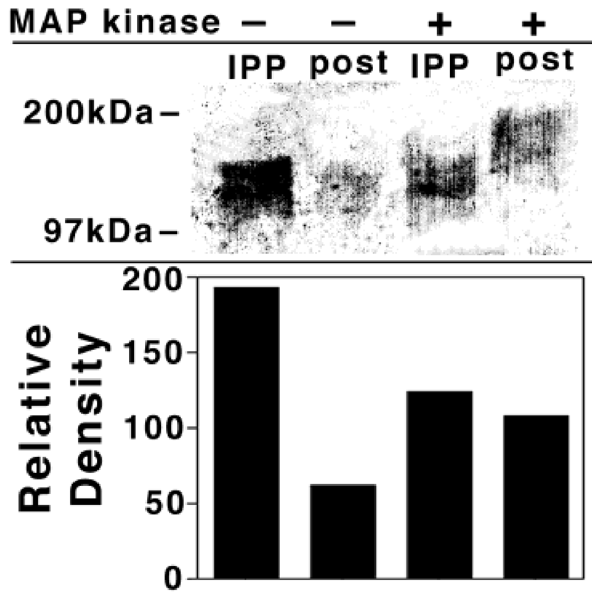

Fig. 5. NF phosphorylation regulates the association of kinesin. Aliquots of the MT motor preparation were incubated with purified MAP kinase as described in Materials and Methods, then immunoprecipitated with anti-kinesin. Immunoprecipitated ("IPP") and non-precipitated ("post") material was subjected to immunoblot analysis with SMI-31. MAP kinase phosphorylated NF-H, as evidenced by slower NF-H electrophoretic migration in MAP kinase-treated samples, and resultant slower migrating isoforms were selectively not co-precipitated by anti-kinesin.

Kinesin Is Preferentially Associated With the "Front" of the Moving Wave of Transporting Subunits

Immunoprecipitation of radiolabeled retinas and axonal segments with anti-kinesin also resulted in a markedly different profile than that obtained with R39 within 2 days following radiolabeling. These differences became increasing obvious at longer times following radiolabeling (e.g., day 3–14); densitometric analysis, as well as visual inspection, of autoradiographs revealed that the distribution of the radiolabeled NF subunit peak co-precipitated by anti-kinesin during continued transport did not exhibit the same broadening as did NFs immunoprecipitated by R39. Rather, the front of the transporting wave of radiolabeled NFs was selectively co-precipitated by anti-kinesin. Indeed, location of the front of radiolabeled subunits immunoprecipitated by R39 matched that of subunits co-precipitated with anti-kinesin (Fig. 7). Moreover, the peak and trail of radiolabeled subunits that were not co-precipitated with anti-kinesin were readily immunoprecipitated following incubation with R39 (Fig. 7). These findings provide additional evidence of a specific association of NFs with kinesin, since the largest amount of radiolabeled NFs are not simply co-precipitated from those segments fractions possessing the largest levels of radiolabeled subunits. Radiolabeled NFs co-precipitated by anti-kinesin eventually exhibited an overall slowing of transport rate during continued transport; however, subunits co-precipitated by anti-kinesin did not

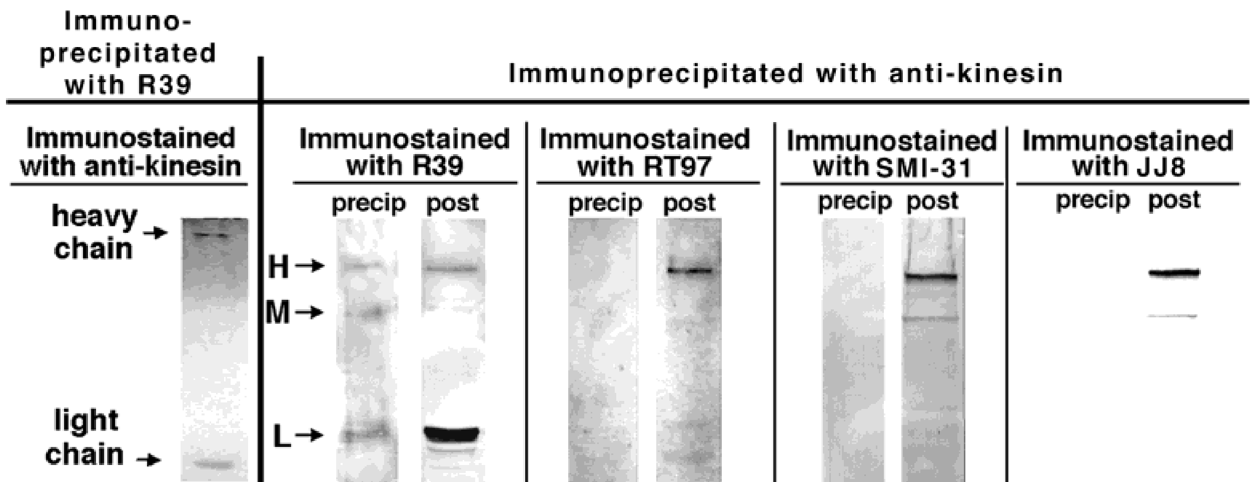

Fig. 6. An anti-kinesin antibody selectively co-precipitates NFs that lack certain C-terminal phospho-epitopes. Immunoblots of material immunoprecipitated ("precip") from homogenates of optic axons by anti-kinesin or R39 along with non-precipitated residue ("post"). Immunostaining with anti-kinesin demonstrated co-precipitation of kinesin by R39, and immunostaining with R39 demonstrated co-precipitation of some subunits by anti-kinesin. However, subunits displaying developmentally delayed C-terminal phospho-epitopes (RT97, SMI-31, JJ8) were selectively not co-precipitated by the anti-kinesin antibody.

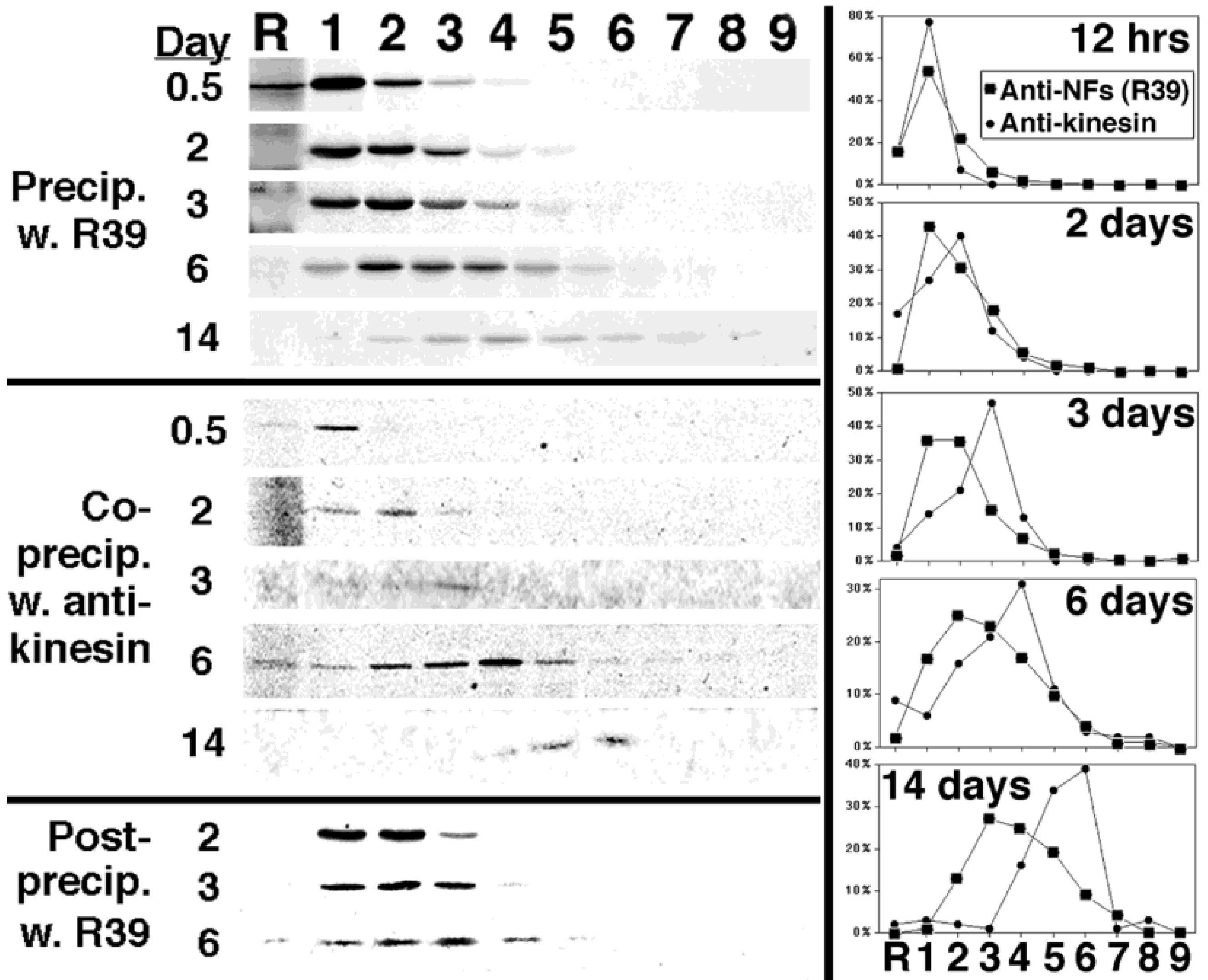

Fig. 7. Selective co-precipitation of the front of the wave of transporting NF subunits with kinesin. Triton-insoluble cytoskeletons from radiolabeled optic axon segments were subjected to immunoprecipitation with R39 or anti-kinesin followed by electrophoresis and autoradiography. The non-precipitated residue following immunoprecipitation with anti-kinesin was re-immunoprecipitated with R39 ("Post-precip. with R39"). NF-L alone is presented to facilitate comparison among samples. From day 2 after injection on, anti-kinesin selectively co-precipitated the front of the wave of radiolabeled NF subunits. Note further that the trail of radiolabeled subunits, not co-precipitated by anti-kinesin, was recovered by re-immunoprecipitation with R39. The accompanying graphs present densitometric analysis of the percentage distribution of NF-L immunoprecipitated from retinas and axonal segments by R39 or anti-kinesin as indicated. Note that, from day 2 after injection on, the peak of radiolabeled NF-L precipitated by anti-kinesin precedes that precipitated by R39.

undergo the regional slowing at segment 3 observed for the overall NF subunit population immunoprecipitated by R39 (Fig. 7) [see also Jung et al., 1998].

A minor population of NF subunits undergo axonal transport in a Triton-soluble form [Tsuda et al., 1997; Jung et al., 1998]. These Triton-soluble subunits migrate at a rate slightly faster than do those incorporated into the Triton-insoluble cytoskeleton. Like that of Triton-insoluble subunits, the front of this wave was also selectively co-precipitated the anti-kinesin antibody, and was recovered a full axonal segment ahead of that from the Triton-insoluble material (Fig. 8). These latter findings provide further evidence of a specific association of NFs with kinesin, since the pattern of Triton-soluble subunits co-precipitated with anti-kinesin differed from those co-precipitated from Triton-insoluble subunits (Fig. 8).

DISCUSSION

Phosphorylation has long been considered to regulate NF axonal transport [Archer et al., 1994; deWaegh et

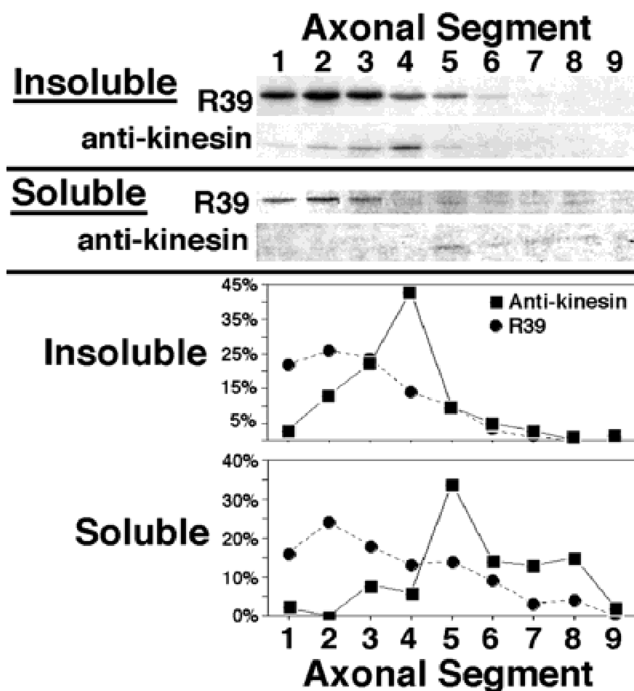

Fig. 8. The front of the transporting wave of radiolabeled Triton-soluble and -insoluble subunits is co-precipitated with anti-kinesin. Triton-soluble and -insoluble material from optic axons 6 days after injection of radiolabel were subjected to immunoprecipitation with R39 or anti-kinesin and electrophoresis and autoradiography. NF-L alone is presented to facilitate comparison among samples. The accompanying graphs present densitometric analysis of the percentage distribution of NF-L as indicated. Note that anti-kinesin selectively co-precipitated the front of the wave of Triton-soluble as well as -insoluble radiolabeled NF subunits.

al., 1992; Hoffman et al, 1983, 1985; Jung and Shea, 1999; Jung et al., 1998; Lasek et al., 1992, 1993; Nixon, 1993, 1998a, b; Watson et al., 1989]. This regulation has been considered to derive at least in part from phospho-dependent interactions of NFs with their putative transport motor. Since the association of NFs with their putative motor system is likely to be reversible, site-specific C-terminal NF phosphorylation been proposed as a potential mechanism to regulate this association [e.g., Baas and Brown, 1997; Nixon, 1998a,b]. This line of reasoning is consistent with continued C-terminal phosphorylation and dephosphorylation of NFs during their transport along optic axons [Komiya et al., 1986; Lewis and Nixon, 1988; Nixon and Lewis, 1986; Nixon et al., 1987, 1994]. The characteristic broadening of the wave of transporting NFs [Archer et al., 1994; Jung et al., 1998; Lasek et al., 1992, 1993; Nixon, 1993, 1998a,b; Watson et al., 1989] has been interpreted to reflect differential association and dissociation of individual NFs with a putative transport motor, i.e., the fastest moving NFs would represent those spending relatively more time in association with the motor, while the slowest moving NFs represent those that have dissociated from the motor for relatively longer periods [Jung et al., 1998; Lasek et al., 1992, 1993; Nixon, 1993, 1998b; Ochs, 1975]. These conclusions are supported by the more rapid axonal transport of less phosphorylated variants, and slowest transport of the most phosphorylated variants, along optic axons [Lewis and Nixon, 1988], and by the slowing of NF transport following experimental phosphatase inhibition in situ [Jung et al., 1998].

We describe herein an association of NFs with the anterograde motor protein kinesin. We further demonstrate that this association is regulated at least in part by NF phosphorylation. These results are consistent with the possibility that kinesin may participate in NF axonal transport. If we consider this to be the case, then dissociation of NFs from kinesin via NF phosphorylation events normally restricted to axons as described herein provides one mechanism by which phosphorylation could contribute to slowing of NF axonal transport as described above. Selective co-precipitation by anti-kinesin of radiolabeled NF subunits from the front, rather than the peak or trail of the transporting wave, is consistent with the notion that slower-moving radiolabeled subunits (i.e., within the trail of the wave) are less frequently associated with kinesin than are the faster-moving subunits (i.e., within the front of the wave). These findings are, furthermore, consistent with the above notion that that fastest-moving subunits achieve their relatively fast rate by spending more time in association with their transport vector than do slower-moving subunits. Assuming that kinesin does indeed mediate certain aspects of NF transport, accumulation of NFs within proximal axons in motor neuron disease could also result from precocious phosphorylation-mediated dissociation of NFs from kinesin [Griffin and Watson, 1988; Julien, 1997; Nixon, 1993].

In these analyses, and in our previous demonstration in cultured neurons and neuroblastoma [Yabe et al., 1999], we have not addressed whether or not the association of kinesin with NFs occurs directly or whether there are one or more "linker" proteins that mediate the association of kinesin with NFs. It is also possible that kinesin is somewhat indirectly involved in NF transport by crosslinking NFs to translocating MTs; however, co-precipitation of NFs from a motor preparation lacking detectable tubulin immunoreactivity argues against this possibility. Experiments with truncated NF subunits will be required for definitive determination of kinesin binding sites on NF subunits. Nevertheless, our studies provide limited speculations regarding the potential location of such binding sites. Our data indicate that (1) kinesin preferentially associates with NF-M and/or NF-H rather than NF-L, and (2) C-terminal phosphorylation events

are correlated with dissociation of NFs from kinesin. Kinesin binding sites could exist on the rod domain, and C-terminal phosphorylation, which enhances extension of NF-M and NF-H sidearms [for review, see Nixon and Shea, 1992], could sterically inhibit the association of kinesin with the rod. However, phosphorylation-mediated sidearm extension may not itself be responsible for inhibiting the association of kinesin with NFs, since NF-H migrating at 200 kDa (and therefore already extensively phosphorylated) [Julien and Mushynski, 1983] was co-precipitated along with kinesin. A kinesin binding site is also unlikely to be within an area of the rod of either NF-H or NF-M that is disclosed following extension of C-terminal sidearms, since phosphorylation would then be expected to promote kinesin binding by exposing such a site. NF-H and NF-M subunits subjected to as yet undisclosed phosphorylation events, characterized herein only by their association with "late-appearing" phospho-epitopes, defined a population of NFs that did not associate with kinesin as readily with NFs lacking such epitopes. These data are consistent with the presence of kinesin binding site(s) on the C-terminal sidearms of NF-M and/or NF-H, and the inhibition of kinesin binding by phosphorylation of (or near) putative binding site(s). Consistent with this possibility is the failure of kinesin to co-precipitate NF-L. Such speculations must be viewed with caution, since kinesin associates with vimentin [Liao and Gundersen, 1998], which may be considered to resemble NF-L more closely than either NF-M or NF-H [for review, see Nixon and Shea, 1992]. Resolution of kinesin binding sites will require further study. It is unlikely that dissociation of NFs from kinesin is due to phosphorylation of kinesin itself, as kinesin phosphorylation enhances, rather than diminishes, the association of kinesin with its cargo [Lee and Hollenbeck, 1995].

Comparative co-purification analyses of vimentin and spinal cord NFs prompted the prior conclusion that NFs did not associate with kinesin [Liao and Gundersen, 1998]. These prior data clearly indicate that relatively less phospho-NFs were associated with kinesin than vimentin. However, our data suggest that the use of spinal cord NFs, which contain a preponderance of extensively phosphorylated subunits, precluded significant association of NFs and kinesin in these prior analyses. While we have not carried out comparative analyses, the kinetics of vimentin:kinesin associations may more closely resemble those of non-phosphorylated NFs with kinesin. Of interest would be to determine the relative affinity of kinesin for vimentin, as well as additional neuronal IFs [for review, see Nixon and Shea, 1992] vs. that of perikaryal and axonal NFs. In addition, during development, doublet NFs consisting of only NF-L and NF-M precede the appearance of triplet NFs containing NF-H [Carden et al., 1987]; of further interest would be to examine the relative affinity of such "immature" vs. "mature" NFs for kinesin.

The assembly state of NF subunits during axonal transport remains controversial. Our demonstration of association of a motor protein with Triton-soluble and -insoluble NF subunits addresses this area in certain respects. While >95% of subunits within axons are polymerized [Morris and Lasek, 1982], Triton-soluble subunits have been shown to undergo axonal transport along the entire length of axons at rates equivalent to and in some cases in excess of those of Triton-insoluble subunits [Jung et al., 1998; Shea et al., 1990]. Such Triton-soluble subunits include a higher percentage of newly synthesized subunits, confirming that they are comprised at least in part of precursors for NF assembly rather than older subunits released by NF catabolism [Jung et al., 1998; Shea et al., 1990]. In addition, transfected NF-M subunits were detected within axons of transgenic mice lacking NFs [Terada et al., 1994]; of particular importance in this latter study is that NF-M cannot form filaments in the absence of NF-L under physiological conditions [Balin et al., 1991; Gardner et al., 1984; Totutake et al., 1984], and these data, therefore, demonstrate transport of non-filamentous subunits. Any proposed transport mechanism should, therefore, encompass the ability to translocate subunits in multiple assembly states. Importantly, however, the vast majority of subunits self-assemble shortly after synthesis and remain as stable polymers once assembled [Black et al., 1986; Morris and Lasek, 1982]. Therefore, a NF motor protein that does not discriminate among assembly states, would nevertheless translocate most subunits in assembled form. A minority of subunits could undergo transport in the form of monomers and/or small oligomers as a consequence of association with a motor prior to NF assembly. This possibility is supported by the co-localization of kinesin with the punctate NF oligomeric assemblies that are prominent within growing axons of cultured neurons [Yabe et al., 1999]. In addition to putative repair/remodeling functions for transported monomers/small oligomers once the axon is established, one could consider the alternative possibility that some unassembled subunits and/or small oligomers may inadvertently continue to undergo translocation. A neuron may simply rely on the rapid kinetics of NF subunit assembly to insure that the vast majority of subunits undergo axonal transport in filamentous form, and need not invoke additional selection mechanisms to preclude transport of potentially inappropriate forms. Experimental conditions in which NFs are reduced or depleted while individual, non-assembling subunits are over-expressed [Terada et al., 1994] would be expected to, and do, highlight translocation of monomers or small oligomers. Similarly, our own

experiments focused on Triton-soluble subunits [Jung et al., 1998; Shea et al., 1990] would be expected to demonstrate, and do demonstrate, that such subunits can indeed undergo axonal transport isolation independently of their incorporation into Triton-insoluble structures. However, such observations may highlight axonal transport of certain NF subunits "by default," rather than revealing the most physiologically important mode of subunit transport. Further in this regard, hypotheses suggesting that monomers and/or small oligomers represent the major form of transported subunits seem intuitive in terms of energy conservation, since the neuron could replace portions of the cytoskeleton rather than continually translocate the entire axonal cytoskeleton. However, to accomplish translocation of monomers/small oligomers, it must be considered that each "unit" of transported material requires its own motor. Taken to the extreme, translocation of monomers would require one motor per subunit, and translocation of small oligomers would require a motor for a relatively small number of subunits, both of which are at odds with considerations of energy conservation. Further analyses of motors and the assembly state(s) of transporting NF subunits will be required to resolve these theoretical considerations.

Implication of kinesin, a motor known to mediate fast axonal transport, in some aspect of the translocation of slow axonal transport constituents such as NFs points towards a continuum between fast and slow transport. This is consistent with the observation of a population of NF subunits undergoing axonal transport at rates in vast excess of those reported previously [Lasek et al., 1993]. The size of cytoskeletal polymers such as NFs vs. fast transport constituents such as vesicles, coupled with NF-NF interactions [e.g., Eyer and Leterrier, 1988; Leterrier et al., 1996; Shea and Beermann, 1994] that are likely to compete with NF-motor interactions, could impart an average slow transport rate to such cytoskeletal constituents even if they utilize a motor that transports other cargo at fast transport rates. Importantly, we do not interpret these data to indicate that kinesin itself moves within slow axonal transport; indeed, prior studies have demonstrated that this is not the case [Elluri et al., 1995]. Rather, we consider, as previously proposed [e.g., Baas and Brown, 1997; Jung and Shea, 1999; Nixon, 1993; Ochs 1975,], that it is the relative length of time that a given protein remains associated with its motor that dictates its relative transport speed. A compelling computer simulation supporting this concept has been presented [Bray and Mills, 1991].

The potential participation of kinesin, and/or any anterograde motor, in NF transport has implications for the mechanism underlying bulk segregation of NFs within axons rather than dendrites: since axonal MTs are oriented uniformly with their plus end distal to the cell body, while dendritic MTs are nonuniformly oriented [Baas et al., 1988; Heidemann et al., 1991], kinesin would reversibly translocate NFs into and out of dendrites, but would uniformly translocate NFs into and along axonal neurites.

Many novel kinesins and members of the kinesin superfamily (KIFs), including some neuronal-specific members, have been described [Aizawa et al., 1992; Hirokawa, 1997]; we have noted an additional 175 kDa kinesin-related protein herein. Elucidation of which kinesin(s) or KIF(s) mediate(s) are associated with NFs, as well as analyses of potentially responsible site-specific C-terminal NF phosphorylation, will contribute to understanding NF axonal transport, and possibly overall slow axonal transport, in health and neurodegenerative conditions.

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