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Myosin Va binding to neurofilaments is essential for correct myosin Va distribution and transport and neurofilament density

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The identification of molecular motors that modulate the neuronal cytoskeleton has been elusive. Here, we show that a molecular motor protein, myosin Va, is present in high proportions in the cytoskeleton of mouse CNS and peripheral nerves. Immunoelectron microscopy, coimmunoprecipitation, and blot overlay analyses demonstrate that myosin Va in axons associates with neurofilaments, and that the NF-L subunit is its major ligand. A physiological association is indicated by observations that the level of myosin Va is reduced in axons of NF-L–null mice lacking neurofilaments and increased in mice overexpressing NF-L, but unchanged in NF-H–null mice. In vivo pulse-labeled myosin Va advances along axons at slow transport rates overlapping with those of neurofilament proteins and actin, both of which coimmunoprecipitate with myosin Va. Eliminating neurofilaments from mice selectively accelerates myosin Va translocation and redistributes myosin Va to the actin-rich subaxolemma and membranous organelles. Finally, peripheral axons of *dilute-lethal* mice, lacking functional myosin Va, display selectively increased neurofilament number and levels of neurofilament proteins without altering axon caliber. These results identify myosin Va as a neurofilament-associated protein, and show that this association is essential to establish the normal distribution, axonal transport, and content of myosin Va, and the proper numbers of neurofilaments in axons.

Introduction

The myosin Va family of nonmuscle myosin genes includes the *dilute* gene from mouse, the *myr6* gene from rat, the *MYO2* and *MYO4* genes from *Saccharomyces cerevisiae*, the brain *myosin Va* gene from chicken, the *Drosophila myosin V* gene, and the *myosin Va* gene from human (Berg et al., 2001). The protein products of these genes consist of a globular head domain sharing ~40% homology with the head domain of myosin II, followed by a myosin I–like neck domain containing six IQ motifs that bind four calmodulin light chains and two light chains (Espreafico et al., 1992). The central stalk-like region contains a myosin II–like α helical coiled-coil domain and a second globular domain of unique amino acid sequence. At least three isoforms of myosin V exist: Va (Mercer et al., 1991), Vb (Espreafico et al., 1992), and Vc (Rodriguez and Cheney, 2002). The distinctive functions of these differentially expressed isoforms are unknown. Myosin Va is the specific focus of our studies.

Proteins of the myosin V family serve as molecular motors to transport diverse molecules. Analyses of the *MYO2* gene

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*Abbreviations used in this paper: *dl*, *dilute lethal*; GFAP, glial fibrillary acidic protein; IF, intermediate filament.

Key words: myosin Va; *dilute*; neurofilaments; NF-associated proteins; axonal transport

implicate yeast myosin V in the assembly and transport of actin, the translocation of specific proteins to sites of polarized growth, and the transport of vesicles (Santos and Snyder, 1997). The yeast *MYO4* gene product transports components necessary for regulating gene expression and targets mRNA molecules to sites of polarized growth (Bobola et al., 1996). In rat and chick neuronal tissue, myosin Va mediates actin-dependent movement of synaptic vesicles and ER (Tabb et al., 1998), and supports filopodial extension in growth cones (Wang and Jay, 1997).

Myosin Va protein in brain is highly expressed (Cheney et al., 1993) and broadly distributed regionally (Mercer et al., 1991; Espreafico et al., 1992), with prominent localizations in growth cones and neurites of cultured neurons, astrocyte processes (Espindola et al., 1992), and axons of several sensory organs (Hasson et al., 1997). Additionally, myosin Va has been reported to immunolocalize to the intermediate filament (IF)* compartment in a variety of cultured cell types (Engle and Kennett, 1994). Although these findings document that myosin Va is a significant component of CNS neurons and sensory organs, its neural functions are still poorly defined.

Mutations in the mouse myosin Va gene, *dilute*, cause not only defective melanosome movement but also profound neurological symptoms, including a convulsive disorder that results in death by 3–4 wk of age (Searle, 1952). Mutations in the human *myosin Va* gene cause Griscelli's disease, a childhood disease characterized by pronounced hypopigmentation and a convulsive disorder that leads to premature death (Pastural et al., 1997). A similar phenotype is associated with mutations of the ashen locus harboring the Rab27a gene (Menasche et al., 2000), which regulates myosin Va recruitment to melanosomes (Wu et al., 2001). Some features of the *dilute* phenotype also result from the flailer mutation, which originates from germ line exon shuffling between the guanine nucleotide binding protein β 5 and myosin Va genes, leading to the synthesis of a mutant hybrid protein that competes with wild-type myosin Va (Jones et al., 2000). These phenotypes clearly underscore the importance of myosin Va for normal neurological function.

In view of the importance of myosin Va in brain and its possible association with IFs (Engle and Kennett, 1994), we investigated whether myosin Va interacts with neurofilaments and how it influences the properties of neurofilaments in neurons. Neurofilaments predominate in axons and, to a lesser extent, in dendrites and perikarya of the nervous system where they modulate the polar shape and size of the neuron (Nixon and Shea, 1992). Unlike other classes of IFs, neurofilament proteins are heteropolymers formed from three distinct subunits with apparent molecular masses of 200 kD (NF-H), 150 kD (NF-M), and 70 kD (NF-L) on SDS–polyacrylamide gels. In some axons, neurofilaments form a three-dimensional network with microtubules and actin filaments, mediated by one or more members of a family of cross-linking proteins (Svitkina et al., 1996; Yang et al., 1996). How the organization of this network is achieved in axons is not known.

Neurofilaments, microtubules, and microfilaments, as well as their associated proteins, are transported from the perikaryon at speeds of 0.1–2.0 mm/d, collectively referred to as the slow phase of axonal transport. This range of rates is distinct from that of fast axonal transport, which carries mainly vesicular constituents by a microtubule-dependent mechanism powered by kinesin in the anterograde direction (Hirokawa et al., 1991) and by dynein in the retrograde direction (Schnapp and Reese, 1989). It remains controversial as to whether different filamentous cytoskeletal structures are transported exclusively as polymers, subunits/oligomers, or in both forms under different conditions (Hirokawa et al., 1997; Wang et al., 2000; Yabe et al., 2001). Short-range transport mechanisms also mediate restricted longitudinal or lateral movement of organelles within a specific compartment of the neuron. Roles for myosin Va in this form of transport have been suggested (Huang et al., 1999); however, the molecular mechanisms responsible for the movements of cytoskeleton components within axons are poorly understood.

In the study presented here, we establish by multiple criteria that the neurofilament is a major ligand for myosin Va in mouse central and peripheral nervous tissue. We demonstrate that myosin Va binds selectively to the NF-L subunit of the filament, and that myosin Va content, transport, and distribution in axons are regulated in vivo, in part, by levels of NF-L. Our further observations show that myosin Va moves in the slow phase of axonal transport, prominently associates with neurofilaments, and alters neurofilament density selectively when deleted in *dilute-lethal* (*dl*) mice, suggesting that myosin Va plays a role in the behavior of neuronal cytoskeletal elements in addition to its well-recognized role in translocating membranous organelles.

Results

Myosin Va is abundant in neurofilament-enriched cytoskeletons

An affinity-purified polyclonal antisera against the carboxy-terminal end of myosin Va (Evans et al., 1997) detected a single

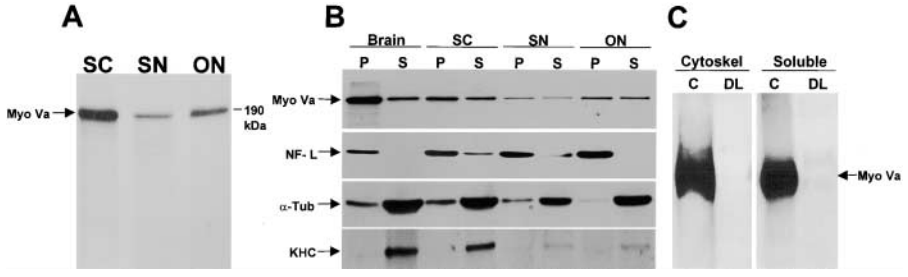

Figure 1. **Distribution of myosin Va in mouse nervous tissues.** (A) Spinal cord (SC), sciatic nerve (SN), and optic nerve (ON) extracts were immunoblotted with myosin Va polyclonal antibody. (B) Myosin Va is abundant in the Triton-insoluble cytoskeletal fraction. Equal amounts of Triton-insoluble (P) and -soluble fractions (S) were immunoblotted with antibodies indicated. (C) Myosin Va is absent in Triton-insoluble (Cytoskel) and soluble fractions of spinal cord from homozygous *dilute* (DL) mouse but not from control mice (C). α-Tub, α-tubulin; KHC, kinesin heavy chain.

Table I. **Relative levels of myosin Va in neuronal tissues**

	Myosin Va
Brain	100
Spinal cord	38.4 ± 5.7
Sciatic nerve	9.6 ± 3.21
Optic nerve	15.5 ± 5.14

Myosin Va is more abundant in CNS tissues than in the PNS. Equal amounts of total protein from spinal cord, sciatic nerve, and optic nerve were immunoblotted with myosin Va polyclonal antibody. Immunoreactive protein bands were quantified using a BioImage whole band analyzer (Kodak) after ECL detection. Each value is a mean ± SD from three experiments.

band of the expected apparent molecular mass (190 kD) in immunoblots of protein from spinal cord, optic, and sciatic nerves (Fig. 1 A). The same protein was identified with a second affinity-purified rabbit polyclonal antiserum raised to the amino-terminal domain of myosin Va (unpublished data). Quantitative immunoblot analysis of total protein extracts from the nervous tissues of the mouse revealed that myosin Va levels are more than twofold higher in the spinal cord than in optic or sciatic nerves (Fig. 1, A and B; Table I). The major proportion of myosin Va (55–67%) immunoreactivity in each of these tissues was associated with the Triton-insoluble cytoskeleton (Fig. 1 B; Table II). By comparison, kinesin heavy chain, another motor protein, was detected only in Triton-soluble fractions (Fig. 1 B). The absence of myosin Va immunoreactive protein in spinal cord fractions from homozygous *dl* mice carrying a mutation in the myosin Va gene (Fig. 1 C) established the specificity of the affinity-purified polyclonal myosin Va antibody and confirmed the identity of the cytoskeleton-associated 190-kD protein from normal mice as myosin Va. For comparison with myosin Va, a known myosin Va ligand (actin) and NF-L were also analyzed by quantitative Western blot on the same tissue fractions. The proportions of actin were roughly comparable in Triton-soluble and Triton-insoluble fractions of the brain and spinal cord. However, in the optic and sciatic nerves, cytoskeletal fractions contained only a third of the total actin, but 55–61% of the total myosin Va (Table II).

Myosin Va colocalizes with neurofilaments in axons

Myosin Va immunoreactivity was broadly distributed in neurons within the mouse central and peripheral nervous systems (Fig. 2), as previously noted. Purkinje cells and their elaborate dendritic arborizations were well immunolabeled, as previously shown (Bridgman, 1999) (Fig. 2 A), and other neuronal populations were strongly immunoreactive (Fig. 2 A, inset). Axons in spinal cord (Fig. 2 B) and sciatic nerve fibers (Fig. 2 C) were also well labeled. By contrast, immunoreactive myosin Va was absent in the corresponding nervous tissue regions of *dl* mice (unpublished data).

Ultrastructural inspection of immunogold-labeled sections of mouse optic axons revealed that myosin Va immunolabeling in axons is most abundant in association with neurofilaments (Fig. 2, D, F, and G). In some cases, trains of anti–myosin Va gold particles traced the outline of a single neurofilament (Fig. 2, F and G). Sections from optic nerves of 21-d-old *dl* mice (Fig. 2 E) showed <6% of the total gold labeling seen in age-matched control mice (Fig. 2 D). Immunoreactivity was notably absent from microtubules and myelin (Fig. 2, D–G, arrowheads). To assess the relative distribution of immunoreactive myosin Va, we counted gold particles on, or immediately adjacent to, specific cellular structures. In 25 micrographs of immunolabeled optic axons, the number of gold particles associated with neurofilaments was approximately fourfold greater than that associated with vesicular organelles and the subaxolemmal compartment. Myosin Va immunoreactivity was also associated with structures previously identified to be sites of myosin Va localization (Bridgman, 1999), including the subaxolemmal membrane (unpublished data), smooth ER (Fig. 2 H), mitochondria (Fig. 2 J), and small, membrane-bound vesicles within nerve terminals or preterminal axon swellings (Fig. 2 I).

Myosin Va associates only with the NF-L subunit of neurofilaments

Because neurofilaments are heteropolymers consisting of three subunits, NF-H, NF-M, and NF-L, we used a blot overlay analysis to investigate which of the neurofilament subunits bind to myosin Va. Purified neurofilament subunits were separated on SDS–polyacrylamide gels and blotted onto nitrocellulose membranes, which were then incubated in solutions containing decreasing concentrations of myosin Va fused with β-galactosidase (Fig. 3 A, lanes 2–5). As a control, purified β-galactosidase protein was incubated with immobilized neurofilament proteins in a concentration equal to the highest concentration of myosin Va–β-gal fusion protein used (Fig. 3 A, lane 6). Bound myosin Va–β-gal or β-galactosidase alone was detected by Western blot analysis using an anti–β-galactosidase antibody. Fig. 3 A demonstrates that myosin Va bound to the NF-L subunit in a concentration-dependent manner, but did not bind to NF-M or NF-H. β-Galactosidase

Table II. **Relative content of myosin Va, NF-L, and actin in Triton-soluble and cytoskeletal-associated fractions of brain, spinal cord, sciatic nerve, and optic nerve**

	Myosin Va		NF-L		Actin	
	Cytoskeletal	Soluble	Cytoskeletal	Soluble	Cytoskeletal	Soluble
Brain	67 ± 5.8	33 ± 5.8	100	0	57.4 ± 3.8	42.6 ± 3.8
Spinal cord	55 ± 7.1	45 ± 7.1	75 ± 14.1	25 ± 14.1	49.1 ± 8.7	50.9 ± 8.7
Sciatic nerve	61 ± 1.2	39 ± 1.2	85 ± 5.3	15 ± 5.3	35.0 ± 7.6	65.0 ± 7.6
Optic nerve	55 ± 0.4	45 ± 0.4	100	0	37.5 ± 6.3	62.5 ± 6.3

Values are percent of total content in the given tissue. Equal amounts of protein from brain, spinal cord, sciatic nerves, and optic nerve were immunoblotted with myosin Va polyclonal antibody (pMyo Va), NF-L (NR-4), or actin (A-2066; Sigma-Aldrich) antibody (Kayalar et al., 1996). Immunoreactivities of the protein bands were quantified using a BioImage whole band analyzer (Kodak) after ECL detection. Each value is an average of three experiments (±SD).

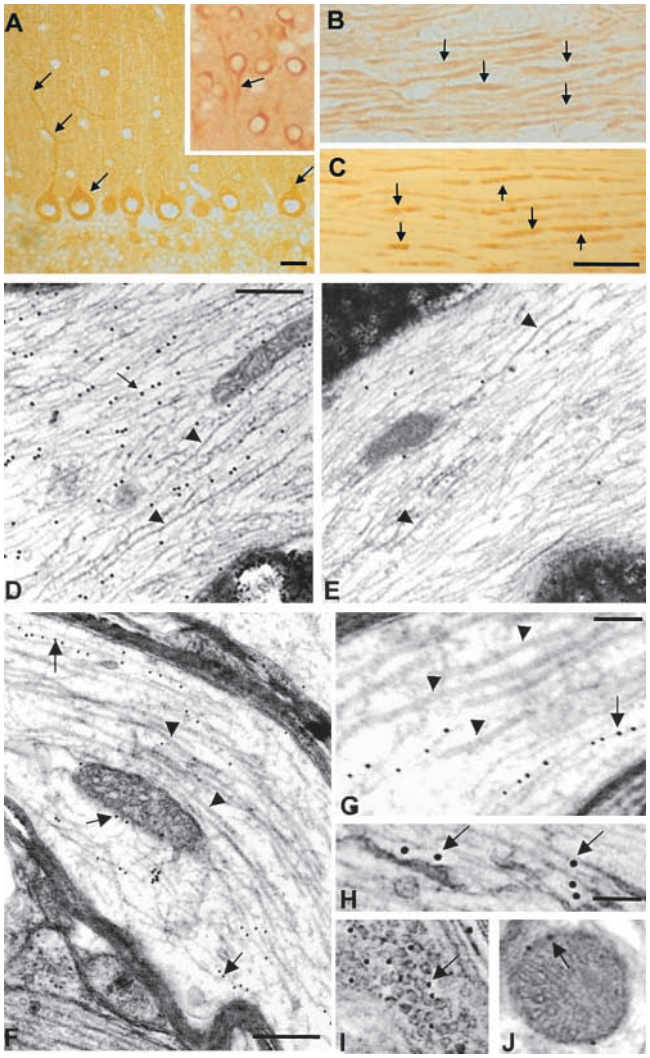

Figure 2. **Distribution of myosin Va immunoreactivity in CNS and PNS tissues.** (A–C) Immunocytochemistry with myosin Va antibody reveals widespread labeling of neuronal populations and fiber tracts in cerebellum (A), cerebral peduncle (A, inset), spinal cord–corticospinal tract (B), and sciatic nerve (C). Bar, 100 μm. (D–J) Ultrastructural localization of myosin Va within axons of mouse brain and optic nerve. Immunogold electron microscope studies of optic nerve (D and E) with myosin Va antibody demonstrate that myosin Va heavily decorates neurofilaments in optic nerves of control mice (D) but not in *dl* mice (E), which lack myosin Va. In axons with fewer neurofilaments, myosin Va is localized to neurofilaments (F and G), and it is rarely associated with microtubules (see arrowheads). Myosin Va is also associated with profiles of smooth ER (H, large arrow), small intracellular vesicles (I, arrow), and mitochondria (J, large arrows). Bars: (D and E) 0.5 μm; (F–J) 2 μm.

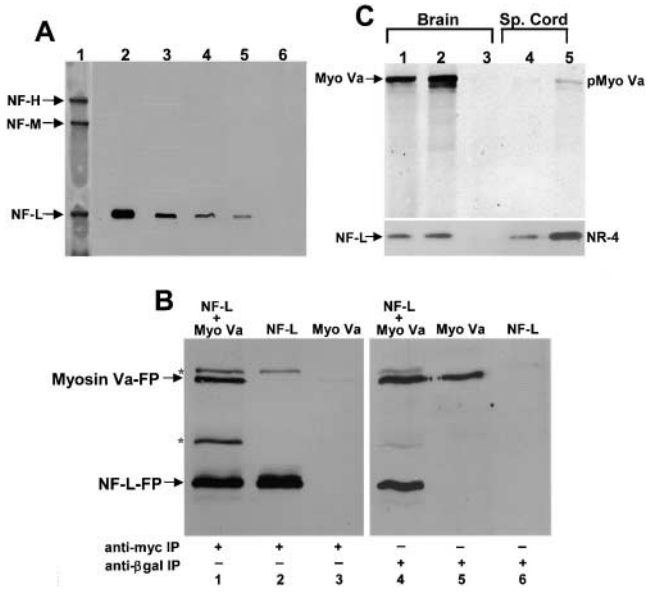

Figure 3. **Myosin Va binds only to NF-L subunit of the neurofilament triplet.** (A) Equal amounts of purified neurofilament subunits (H, M, and L) were blotted onto membranes and incubated with different concentrations of recombinant myosin Va tagged with β-galactosidase (lane 2, 10 μg; lane 3, 1 μg; lane 4, 0.1 μg; lane 5, 0.01 μg; and lane 6, 0 μg). Binding of myosin Va to NF-L was detected with an anti–β-gal antibody. Lane 1 is Coomassie blue staining of purified NF subunits. (B) Myosin Va and NF-L fusion proteins physically associate with each other in coimmunoprecipitation assays. Bacterial extracts containing either a myosin Va–β-gal fusion protein (myosin Va–FP) or an NF-L–c-Myc fusion protein (NF-L–FP) were mixed together to allow binding. Resultant NF-L–FP–myosin Va complexes were precipitated with either an anti–c-myc polyclonal antisera or an anti–β-galactosidase monoclonal antibody. Asterisks indicate nonspecific binding. (C) In vivo evidence for a physical interaction between NF-L and myosin Va. Triton-insoluble cytoskeleton preparations from brain and spinal cord at protein amounts of 0.6 mg (lane 4) and 1.2 mg (lanes 1 and 5) or 2.4 mg (lane 2) were immunoprecipitated with anti–NF-L antibody (NR-4). Lane 3 contained 2.4 mg of brain protein without antibody. The precipitates were immunoblotted with antibodies to myosin Va or NF-L (NR-4). The immunoprecipitates contained a strong immunoreactive myosin Va band at 190 kD on the gel and minor degraded forms.

alone did not bind (Fig. 4 A, lane 6), indicating that the association of myosin Va–β-gal fusion protein with NF-L is due to the myosin Va portion of the fusion protein.

Myosin Va fusion protein coimmunoprecipitates with NF-L fusion protein from bacterial extracts

To confirm the specificity of the association between myosin Va and the NF-L subunit, we expressed a cloned myosin Va–β-galactosidase fusion protein (myosin Va–FP) or a cloned NF-L–c-Myc fusion protein (NF-L–FP) in bacteria. Crude extracts prepared from each expression system were mixed together to allow binding between myosin Va and NF-L, which were then immunoprecipitated with an antibody to either myosin Va–FP (anti–β-gal monoclonal antibody) or NF-L–FP (anti-Myc polyclonal antibody). The immunoprecipitates were separated on SDS-PAGE and blotted onto nitrocellulose for Western blot analysis. Myosin Va–FP and NF-L–FP were detected with a mixture of anti–β-gal and anti–c-myc antibodies. Anti-Myc Ab (Fig. 3 B, lane 1) or anti–β-gal Ab (Fig. 3 B, lane 4) immunoprecipitated both myosin Va–FP and NF-L–FP. As expected, in unmixed bacterial extracts, Myc antibody immunoprecipitated only NF-L–FP (Fig. 3 B, lane 2) but not myosin Va–FP (Fig. 3 B, lane 3), whereas β-gal antibody specifically precipitated myosin Va–FP (Fig. 3 B, lane 5) but not NF-L–FP (Fig. 3 B, lane 6). In contrast to NF-L, bacterially expressed NF-M or NF-H subunits alone did not coimmunoprecipitate with myosin Va–FP (unpublished data).

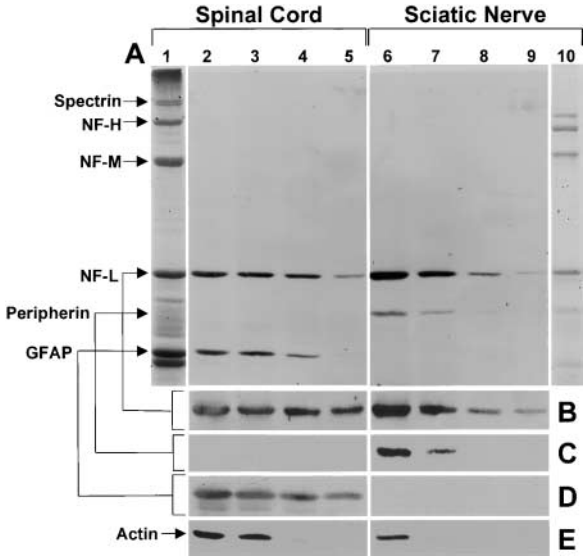

Figure 4. **Myosin Va selectively binds to IF proteins in blot overlay assays.** Cytoskeletal preparations (lane 2, 158 μg; lane 3, 100 μg; lane 4, 50 μg; lane 5, 25 μg; lane 6, 100 μg; lane 7, 50 μg; lane 8, 25 μg; and lane 9, 12.5 μg) from (A) spinal cord (lanes 1–5) and sciatic nerves (lanes 6–10) of C57BL/6J mice were fractionated on 7% polyacrylamide gels and transferred to membranes, and blot overlay assay was done with purified myosin Va–tagged protein, as indicated in Fig. 3 A. Myosin Va also bound to peripherin and GFAP. GFAP is present in spinal cord cytoskeletons (lanes 2–5) but not in sciatic nerves (lanes 6–9). Peripherin is expressed in sciatic nerve (lanes 6–9) but not in spinal cord. Lane 1, spinal cord; lane 10, sciatic nerve cytoskeletal proteins stained with Coomassie blue. The following panels were probed with the indicated antibody to show their identity: B, NF-L; C, peripherin; D, GFAP; and E, actin.

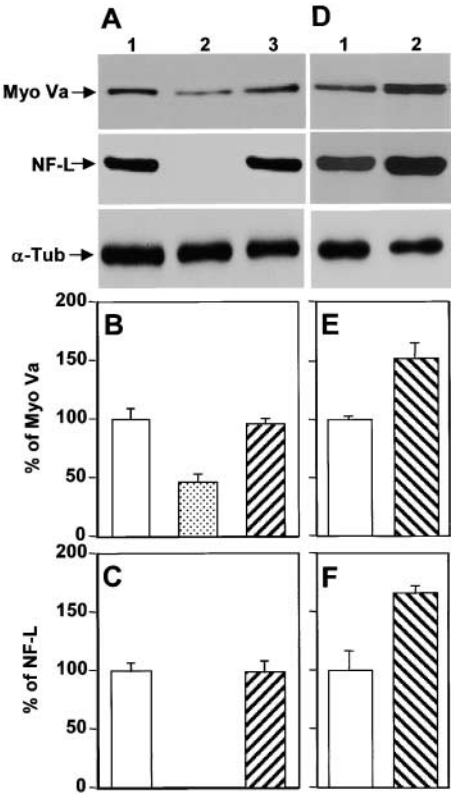

Figure 5. **Myosin Va levels are modulated by NF-L expression in sciatic nerves.** (A) Myosin Va levels are significantly lower in sciatic nerves from NF-L–deleted mice. Equal amounts of sciatic nerve cytoskeletal protein from wild-type (lane 1), NF-L–deleted (lane 2), and NF-H–deleted mice (lane 3) were immunoblotted with antibodies to either myosin Va or NF-L (NR-4). Quantitative results for the expression of myosin Va (B) and NF-L (C) in control mice (open bars); NF-L null (dotted bars) and NF-H null (striped bars) are also shown. In D, myosin Va levels are increased in sciatic nerves from NF-L–overexpressing mice. Cytoskeleton fractions from sciatic nerves of wild-type (D, lane 1) and NF-L–overexpressing mice (D, lane 2) were immunoblotted as indicated in A. The quantitative values for the expression of myosin Va (E) or NF-L (F) in control (open bar) and NF-L transgenic mice (striped bar) are shown. Myosin Va and NF-L levels in NF-L transgenic mice are expressed relative to values for control mice. α-Tub, α-tubulin.

Interaction of endogenous myosin Va with NF-L

Because the NF-L subunit of the neurofilament triplet is the prime ligand for myosin Va, it was of interest to determine whether myosin Va would coimmunoprecipitate with NF-L from neuronal tissue. Using the NF-L mAb NR-4 (Sigma-Aldrich), we immunoprecipitated NF-L from Triton-insoluble cytoskeletal preparations of brain and spinal cord and observed that the immunoprecipitates were enriched not only for NF-L (Fig. 3 C, bottom) but also for myosin Va (top, lanes 1, 2, 4, and 5). In the absence of NF-L antibody, no myosin Va or NF-L sedimented after centrifugation of the Sepharose beads (lane 3), indicating that precipitation of myosin Va and NF-L was antibody dependent.

Myosin Va binds selectively to IF proteins of several classes

The specificity of myosin Va binding to cytoskeletal proteins was further investigated by blot overlay analysis of Triton-insoluble cytoskeletal fractions from mouse spinal cord (Fig. 4 A, lanes 1–5) and sciatic nerve (Fig. 4 A, lanes 6–10). In both tissues studied, the major binding partner of myosin Va was NF-L (Fig. 4 B); however, we also observed binding of myosin Va to a 45-kD protein in spinal cord (Fig. 4 A, lanes 2–5) and to a 55-kD protein in sciatic nerve (Fig. 4 A, lanes 6–9). Based on these apparent molecular masses on SDS gels, we probed the same blots with antibodies to glial fibrillary acidic protein (GFAP), a 45-kD protein (Fig. 4 D), and peripherin (Fig. 4 C), a 55-kD protein, and observed that the immunoreactivity of these proteins overlapped precisely with the positions of the protein bands detected by myosin Va binding. Thus, from the dozens of different proteins present in the cytoskeleton preparations, myosin Va bound specifically to three structurally related IF proteins. It is not surprising that actin was not detected in this overlay assay because this myosin Va clone (Fb8; Engle and Kennett, 1994) does not have a complete actin binding site.

Myosin Va levels in sciatic nerve are influenced by neurofilaments in vivo

Based on the foregoing evidence of a myosin Va–NF-L association, we next examined whether the levels of NF-L in axons influence the axonal content of myosin Va by analyzing mice in which neurofilament levels were altered. Targeted mice lacking the NF-L gene are devoid of NF-L and virtually all neurofilaments (Zhu et al., 1997). NF-L is absent in

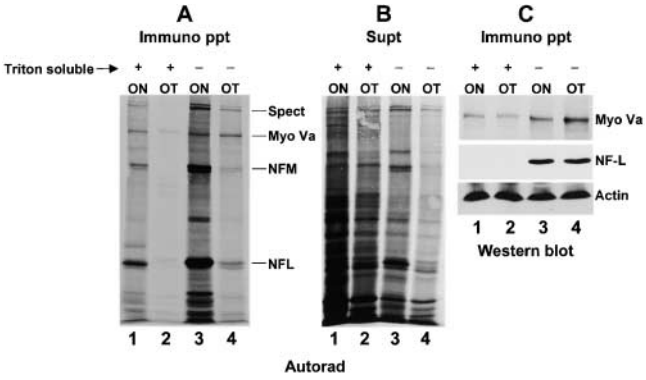

Figure 6. **A pool of pulse-labeled myosin Va in optic axons is associated with the neurofilament cytoskeleton.** Pulse-labeled NF-H–deleted optic nerves (lanes 1 and 3) and optic tracts (lanes 2 and 4) were isolated and fractionated. (A) Triton-soluble (lanes 1 and 2) and -insoluble (lanes 3 and 4) fractions were immunoprecipitated with myosin Va antibody. The washed immunoprecipitates (A) and the corresponding "unprecipitated" supernatants (B) were separated on SDS gels, transferred to nitrocellulose membranes, and visualized by autoradiography. Blots from A were then probed with antibodies to myosin Va, NF-L, and actin to confirm the positions of these proteins on the gels. Note that absence of soluble NF-L explains the lack of immunostaining in immunoprecipitates of Triton-soluble fractions. Spect, spectrin.

the sciatic nerves of these mice (Fig. 5, A and C, lane 2), and we observed a 55% reduction in the levels of myosin Va (Fig. 5, A and B, lane 2). By contrast, myosin Va levels were not altered in sciatic nerves of NF-H–deleted mice (Fig. 5, A and C, lane 3), in which NF-L and neurofilament levels in axons are normal (Rao et al., 1998). The effects on myosin Va of increasing NF-L levels and filament number in axons were also examined in mice overexpressing the NF-L protein (Xu et al., 1993). NF-L levels and neurofilament counts in sciatic nerves from NF-L–overexpressing mice have been previously shown to be ~1.5-fold higher than the corresponding control mice (Xu et al., 1993). We confirmed the reported increase in NF-L levels (Fig. 5, D and F, lane 2) and observed by quantitative immunoblot analyses that myosin Va levels were comparably increased (50%) in the sciatic nerves of NF-L transgenic mice (Fig. 5 D, lane 2). These results indicate that axonal myosin Va content is influenced by levels of NF-L, neurofilament number, or both.

In vivo axonal transport of myosin Va

In earlier studies, we tentatively identified a Triton-soluble radiolabeled protein advancing 1–2 mm/d in the slow phase of axonal transport as a form of myosin (Lewis and Nixon, 1988). This protein, however, comigrated on SDS gels with multiple phosphovariants of NF-H, which precluded its full characterization. To eliminate NF-H as a confounding variable, we conducted the present myosin Va transport study in NF-H–deleted mice (Rao et al., 1998). Retinal ganglion cells of NF-H–deleted mice were labeled by intravitreous injection of [^{35}S]methionine, and after 3 d, we prepared Triton-soluble and Triton-insoluble fractions from optic nerves or optic tracts pooled from three mice and immunoprecipitated myosin Va. Myosin Va coimmunoprecipitated with only a limited set of labeled proteins, which included NF-L,

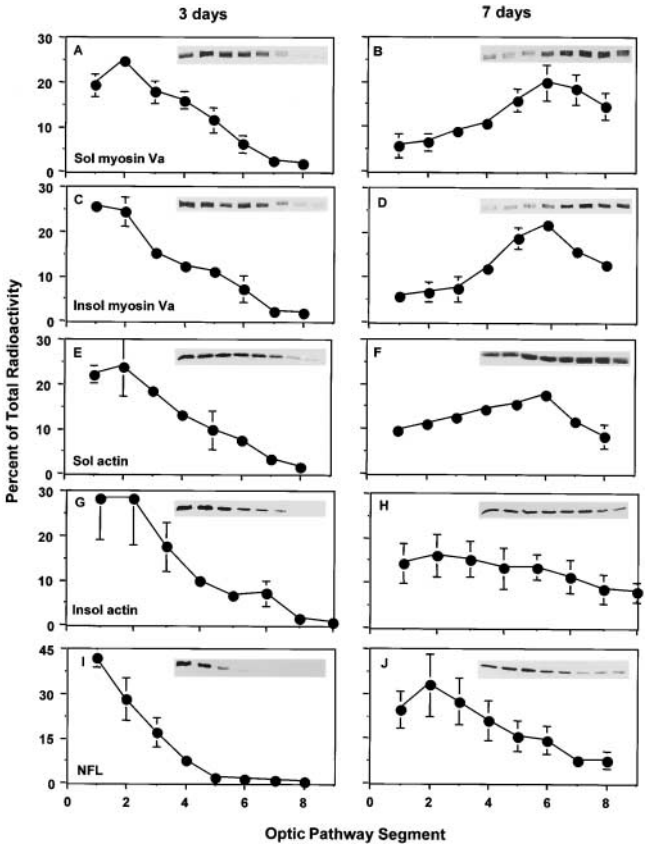

Figure 7. **Quantitative distribution of radiolabeled myosin Va, NF-L, and actin along optic axons at 3 and 7 d after synthesis.** NF-H–null mice were used to determine the distribution of labeled Triton-soluble and -insoluble myosin Va in the optic axons. The optic pathways from multiple groups of three mice at 3 (A, C, E, G, and I) and 7 d (B, D, F, H, and J) after intravitreal injection of [^{35}S]methionine were cut into eight 1.1-mm segments at consecutive levels extending from the eye to the lateral geniculate body. Triton X-100–soluble and –insoluble fractions were subjected to SDS-PAGE, electroblotting, and autoradiography. The regions of the membrane containing labeled soluble (A and B) and insoluble (C and D) myosin Va, soluble (E and F) and insoluble (G and H) actin, and NF-L (I and J) were identified by immunoblotting, and the corresponding radiolabeled bands were quantified by laser densitometry and plotted against optic pathway nerve segments numbered consecutively from the level of the eye. Each point is the mean ± SD for an average of six independent experiments. Note that the error bars for some values are too small to be visible on the graph.

NF-M, spectrin (a known neurofilament binding protein; Frappier et al., 1991), actin, as well as several unidentified proteins (Fig. 6 A). The identities of immunoprecipitated myosin Va, NF-L, and actin were confirmed by Western blot analysis on the same blots after autoradiography (Fig. 6 C). No radiolabeled proteins were precipitated by preimmune IgG used under the same conditions before myosin Va immunoprecipitation (unpublished data).

Most of the radiolabeled myosin Va was still present in the optic nerve 3 d after isotope injection, indicating that myosin Va principally moves in the slow phase of axonal transport. To establish the transport pattern more precisely, additional NF-H–null mice were injected intravitreously with [^{35}S]methionine. After 3 or 7 d, the optic pathways

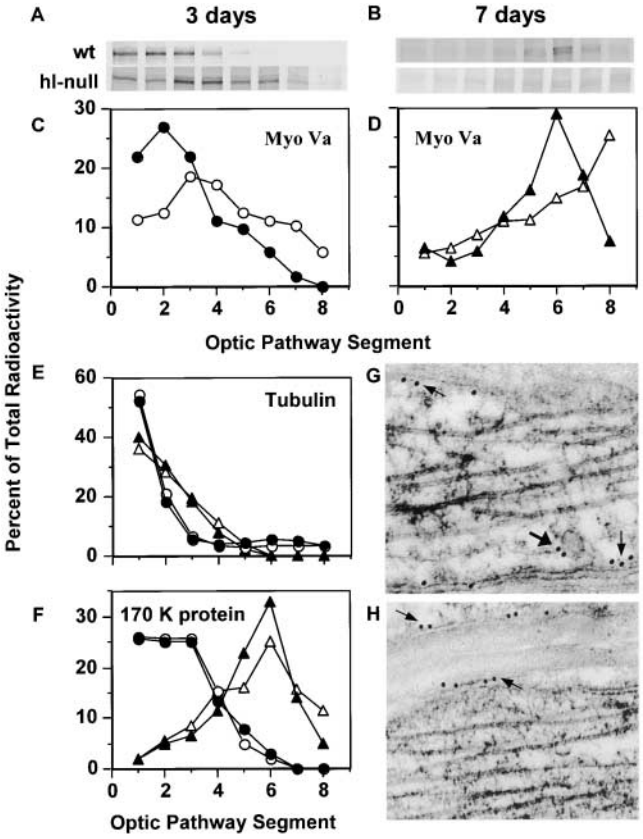

Figure 8. **Axonal transport and distribution of myosin Va along optic axons of mice lacking neurofilaments.** Accelerated axonal transport of radiolabeled myosin Va (A–D), but not tubulin (E) or a 170-kD protein (F) in slow component b, and redistribution of myosin Va (G and H) along optic axons of mice lacking neurofilaments. The optic pathways were dissected from multiple groups of three wild-type (WT) or NF-H/NF-L double null (HL-DKO) mice at 3 and 7 d after intravitreal injection of [^{35}S]methionine and analyzed, as indicated in Fig. 7. (G and H) Immunogold electron microscopy with myosin Va antibody reveals in optic axons of NF-L–null mice no immunolabeling of microtubules and core axoplasm. Myosin Va labeling is restricted to the subaxolemmal compartment (G and H, thin arrows) and membranous organelles (G, thick arrow). Note that the myelin sheaths are negatively stained in these images.

from three mice were then cut into eight consecutive 1.1-mm segments, which were fractionated into Triton-soluble and -insoluble fractions and subjected to electrophoresis, electroblotting, and autoradiography. The myosin Va in Triton-soluble (Fig. 7, A and B) and cytoskeleton-associated fractions (Fig. 7, C and D) displayed a similar transport pattern, moving as a wave averaging 0.73–0.94 mm/d (peak) to 2.2 mm/d (front), which correspond to a typical slow component b rate of transport (0.8–2 mm/d) in optic axons (Nixon, 1991).

Triton-soluble actin displayed a transport profile (Fig. 7 E) similar to that of myosin Va. Cytoskeleton-associated actin also moved into optic axons at a rate of 1.1 mm/d (Fig. 7 G) (Yuan et al., 2000), but a significant proportion of this insoluble actin slowed to a rate similar to that of NF-L (Fig. 7 I, 0.31 mm/day). The slower movement of neurofilaments than much of its associated protein myosin Va suggests a dynamic association during axonal transport, which is not unexpected in light of observations that microtubule-associated proteins, the family of proteins that associate with microtubules in axons, are also transported more rapidly than tubulins in vivo (Nixon et al., 1990; Mercken et al., 1995).

Neurofilament depletion alters the transport and distribution of myosin Va in axons

Direct evidence for an in vivo interaction of myosin Va with NF-L was sought by investigating whether the elimination of neurofilaments altered the movement or principal location of myosin Va in axons. To visualize radiolabeled myosin Va on gels in pulse-labeling studies, we analyzed axonal transport patterns in optic nerves of mice deleted of both NF-L and NF-H. In these neurofilament-deficient axons, the average rate of myosin Va translocation was significantly faster than that in normal axons at 3 or 7 d after [^{35}S]methionine injection (Fig. 8, A and B). Transport of tubulin and major slow component b proteins was unaltered in neurofilament-deficient mice (Fig. 8, E and F). Immunogold labeling NF-L-null mice showed that, in the absence of neurofilaments, myosin Va distribution is restricted to the subaxolemmal compartment and to the surfaces of membranous vesicles (Fig. 8, G and H). An increased incidence of subaxolemmal labeling implied that a proportion of the neurofilament-associated myosin Va was redistributed in these mice. These observations demonstrate unequivocally that a significant proportion of myosin Va normally interacts with neurofilaments in vivo.

Neurofilament number and density are increased in mice lacking myosin Va

To further establish the physiological significance of the myosin Va–neurofilament interaction, we analyzed the ultrastructure and cytoskeletal protein content of peripheral nerves from *dilute* mice and their normal littermates. Neurofilament number was increased nearly twofold ($P < 0.001$) in axons of *dl* mice compared with littermate controls (mean ± SEM, 1177 ± 105 vs. 666 ± 51 NFs/axon, respectively [n = 75–85 axons]). Regression analyses indicate that the density of neurofilaments was also significantly higher ($P < 0.0001$) in axons of all caliber sizes in the *dilute* mice (251 ± 13 vs. 113 ± 7 NF/μm^2) (Fig. 9, A and C, inset). Western blot analyses confirmed an increased content of NF-L of ~60% in *dl* sciatic nerves compared with controls (Fig. 9 E). By contrast, levels of β_{III}-tubulin and actin in the same nerve extracts were not significantly altered by deletion of myosin Va. The distribution of axon cross-sectional areas in *dilute* mice was similar to control mice, indicating that the higher neurofilament densities did not alter axonal calibers (Fig. 9 D). Coupled with the analyses of NF-L–null and transgenic mice (Fig. 5), these studies provide strong evidence for a physiological interaction between myosin Va and neurofilaments, which modulates neurofilament organization in axons.

Discussion

Our results identify the molecular motor, myosin Va, as a neurofilament-associated protein and establish NF-L as a

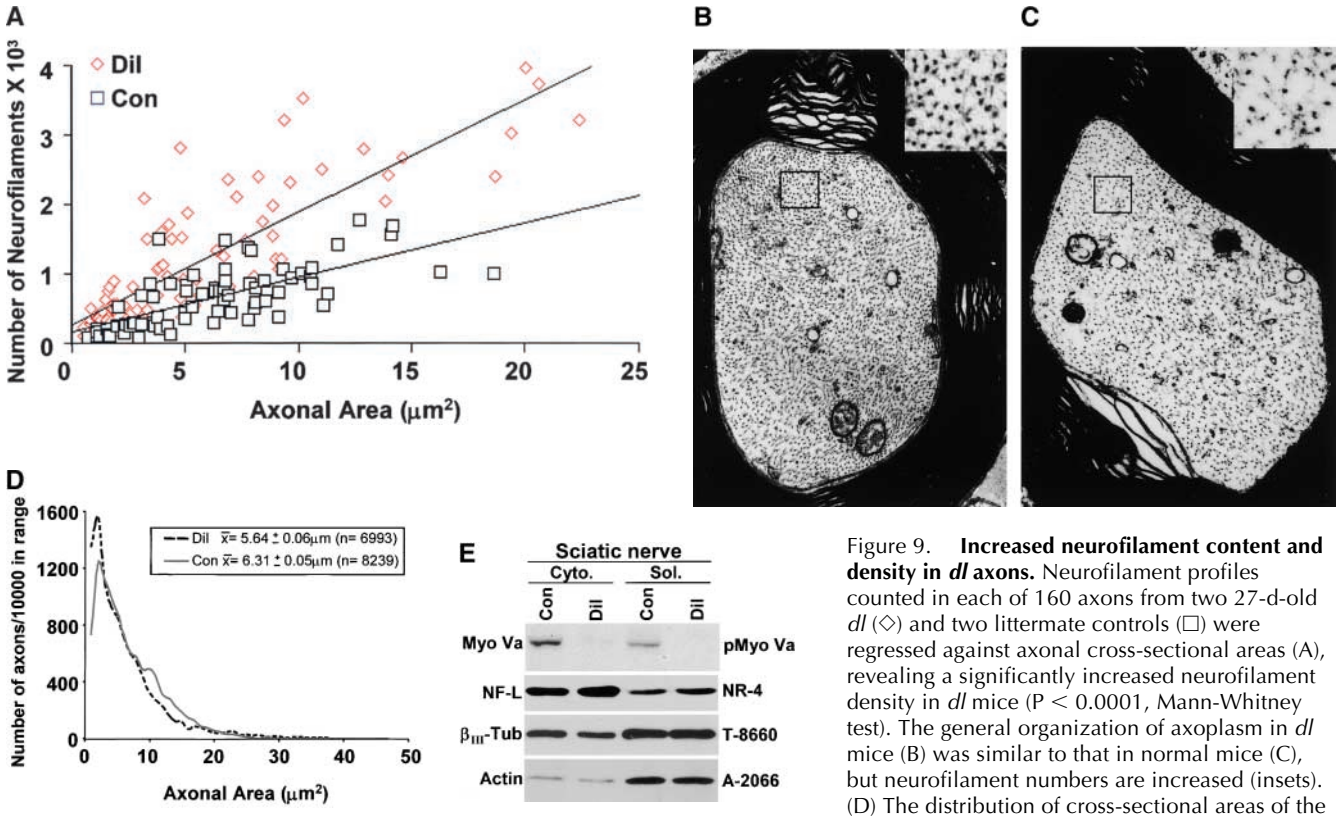

Figure 9. **Increased neurofilament content and density in *dl* axons.** Neurofilament profiles counted in each of 160 axons from two 27-d-old *dl* (◇) and two littermate controls (□) were regressed against axonal cross-sectional areas (A), revealing a significantly increased neurofilament density in *dl* mice ($P < 0.0001$, Mann-Whitney test). The general organization of axoplasm in *dl* mice (B) was similar to that in normal mice (C), but neurofilament numbers are increased (insets). (D) The distribution of cross-sectional areas of the entire fiber population of the sciatic nerves from the four mice analyzed in A. Mean cross-sectional areas (±SEM) are indicated. In E, equal amounts of cytoskeletal (Cyto.) and Triton-soluble (Sol.) fractions from control (Con) and *dl* (Dil) mice were immunoblotted with the antibodies against myosin Va (pMyo Va), NF-L (NR-4), neuron-specific β_{III}-tubulin (T-8660), and actin (A-2066).

major ligand of myosin Va in nervous tissue. Multiple lines of evidence support these conclusions. First, more than half of the total myosin Va in CNS tissues coisolates with the neurofilament-rich Triton-insoluble cytoskeleton. Second, immunoreactive myosin Va is abundant in nerve fiber tracts of brain, spinal cord, and peripheral nerves. Immunoelectron microscopic analyses show that the majority of the myosin Va in axons is associated with neurofilaments. Third, electroblot overlay analyses and in vitro and in vivo coimmunoprecipitation studies with either myosin Va or NF-L antibodies establish that the binding of myosin Va to neurofilaments is mediated through a specific association of myosin Va with the NF-L subunit. Fourth, we demonstrate in vivo that myosin Va levels, distribution, and transport in axons are influenced by the level of NF-L subunits. Finally, deletion of the myosin Va gene in *dilute* mice selectively alters neurofilament content and organization in axons.

In addition to establishing a novel myosin Va–neurofilament association, our results confirm previously observed associations of myosin Va with ER membranes, synaptic vesicles (Tabb et al., 1998), and actin. Extending observations that myosin Va is an actin binding protein (Espreafico et al., 1992; Cheney et al., 1993), we demonstrated that in vivo–labeled actin and myosin Va coimmunoprecipitate and cotransport and that myosin Va antibodies decorate the actin-rich subaxolemmal compartment (Fig. 2; Fig. 8, G and H; Kobayashi et al., 1986). It is not surprising that the myosin Va–neurofilament interaction has been less well appreciated than the associations of myosin Va with actin or membranous organelles because previous myosin Va localization studies have focused on organelle-rich and neurofilament-poor cellular compartments in cell bodies and dendrites in brain, rather than in axons.

The NF-L subunit is one of only a limited number of proteins that interacts directly with myosin Va. The further observation that the two other major ligands in the spinal cord and sciatic nerve are also IF proteins, GFAP and peripherin, suggests that myosin Va plays a more general role in IF behavior or that IFs mediate important functions of myosin Va. By performing multiple protein sequence alignment with hierarchical clustering (Corpet, 1988; Multalin version 5.4.1) on NF-L, peripherin, and GFAP, we found 132 amino acid homologies in a sequence of 350 residues corresponding to the head and rod domains. The observation that as many as 82 of these amino acid positions differ in NF-H and NF-M may partly explain the relative selectivity of the myosin Va binding to the three "core" subunits of IFs. The observation that other high abundance proteins, such as tubulin, bound negligible amounts of myosin Va underscores the specificity of the myosin Va–IF interaction. Moreover, that another molecular motor, kinesin, is found in negligible amounts in cytoskeletal fractions indicates that tight association with cytoskeletal structures is not a general feature of motor molecules.

The association of myosin Va with neurofilaments raises new possibilities regarding its function in the nervous system

where levels are exceptionally high (Cheney et al., 1993). One of these possibilities is modulating the organization of the axoplasm. We observed that myosin Va deletion in *dilute* mice creates a denser packing of neurofilaments in axons, suggesting a role in neurofilament spacing. Because NF-L and actin bind to myosin Va at separate sites, myosin Va may be capable of dynamically cross-linking IFs and microfilaments. Recent studies have emphasized the role of molecules other than neurofilaments themselves in regulating lateral spacing of filaments in axons. Molecules, including BPAG and plectin, have recently been shown to cross-link IFs, microfilaments, and microtubules (Svitkina et al., 1996; Yang et al., 1996), although, unlike myosin Va, these proteins have no known ATPase or motor activity. If myosin Va does in fact link filament systems, it is likely to be in the service of dynamically rearranging these structures within the cytoskeletal network. Neurofilaments could either act as an anchor from which myosin Va could move other proteins or vesicular organelles (e.g., actin) or as a cargo of myosin Va. In regard to the first possibility, neurofilaments provide a three-dimensional lattice interconnecting the microtubule system with the subaxolemmal compartment (Yang et al., 1996). This stationary network of filaments conceivably could represent a system of tracks well suited for myosin Va to guide microfilaments or membranous organelles laterally within axons to achieve the proper radial organization of these structures. Neurofilaments have also been shown to be possible ligands of membrane-associated enzymes and receptors (Terry-Lorenzo et al., 2000; Kim et al., 2002), raising the possibility that movements of molecules of this type along neurofilaments may be mediated by myosin Va. Myosin Va has been implicated in moving actin short distances within growth cones (Evans et al., 1997; Bridgman, 1999; Huang et al., 1999). Finally, short-range rearrangements of neurofilaments within the axon, such as those that occur during early postnatal development (Sanchez et al., 1996), might also require motor activity.

Actin and neurofilaments also move long distances by slow axonal transport. The average rate of myosin Va transport that we observed along optic axons is similar to that of actin and close to the rate at which purified myosin Va moves along actin cables from dissected Nitella cells in vitro (2.5–3.8 mm/d) (Cheney et al., 1993). Interestingly, Willard (1977) identified two polypeptides (195 kD and 200 kD) that cosediment with actin in an ATP-reversible way and were transported along optic axons at slow transport rates. Transport of at least some neurofilaments in growing axons of cultured neurons involves a series of rapid movements punctuated by long periods of immobility (Wang et al., 2000). This pattern suggests that motors, such as kinesin, which are capable of mediating fast transport rates, may be attractive candidates for powering neurofilament movement. Because myosin Va can bind directly to kinesin (Huang et al., 1999), the interaction of myosin Va with neurofilaments could represent one mechanism to facilitate neurofilament movement along microtubules. Although the results in *dilute* mice imply that myosin Va is not essential for movement of neurofilaments into axons, the considerably increased neurofilament number in axons could reflect impaired slow transport. However, a particular pattern of neurofilament distribution along axons, by itself, is not predictive of transport kinetics. An increased rate of incorporation of transported neurofilaments into the stationary cytoskeletal network along axons (Nixon, 1998) would yield a similar picture. Definitive tests of these possibilities require long-term labeling studies that are precluded in *dilute* mice by the frailty and early death of these mice after 3–4 wk.

In conclusion, these novel interactions of myosin Va with IFs indicate previously unrecognized roles for myosin Va in regulating cytoskeleton dynamics in the nervous system. Although long or short range transport and/or rearrangements of neurofilaments represent several of the possible roles, interactions between neurofilaments and myosin Va might instead, or in addition, be important in modulating myosin Va–mediated movements of other cytoskeletal proteins, membranous organelles, or membrane-associated proteins. A variety of experimental approaches will be required to investigate the range of intriguing possibilities.

Materials and methods

Animals and dissections of tissue

Control mice were the normal mouse strain C57BL/6J. Brain, spinal cord, sciatic, and optic nerves from 4-mo-old mice were dissected as previously described (Nixon and Logvinenko, 1986). NF-L–null mice were screened according to Zhu et al. (1997). NF-L Tg mice were screened according to Xu et al. (1993). NF-H–deleted mice were screened according to Rao et al. (1998). NF-H/NF-L double knockout mice (HL-DKO) were generated by crossbreeding and screening according to Rao et al. (1998) and Zhu et al. (1997). *Dilute* mice were bought from Jackson ImmunoResearch Laboratories. All the tissues were frozen on dry ice and stored at −80°C. To extend survival of *dl* mutants to 4 wk, pups were fed manually every 8 h from postnatal day 10 with kitten formula (KMR; PetAg Inc.) dissolved in sterile water and mixed with maize syrup.

Protein isolation, Western blot analysis, and densitometry

Total protein homogenates from brain, spinal cord, sciatic, and optic nerves were made according to Rao et al. (1998). Protein concentration was determined using bicinchoninic acid (BCA) assay kit (Pierce Chemical Co.). Protein extracts of known amounts from different neuronal tissues were fractionated on SDS-PAGE gels containing 6 or 7.5% polyacrylamide and transferred to nitrocellulose membranes. Triton-insoluble and -soluble fractions were made according to Nixon et al. (1990). After protein estimation, equal amounts of Triton-insoluble and -soluble fractions were boiled, run on a 7% SDS–polyacrylamide gel, and immunoblotted with affinity-purified polyclonal antibody directed against the COOH terminus of the myosin Va fusion protein (Evans et al., 1997), NF-L (NR-4), α-tubulin (DMIA), and kinesin heavy chain mAbs as specified by the manufacturer. The blots were processed with the ECL system (Amersham Biosciences) or the alkaline phosphatase system (Promega). Band images were quantified with a BioImage whole band analyzer from Kodak.

Immunocytochemistry of mouse tissues

C57BL/6J mice were anesthetized and fixed by cardiac perfusion using 10% neutral buffered formalin in TBS, pH 7.4. The brain, cervical spinal cord, and sciatic nerves were dissected and 40-μm-thick vibratome sections were processed for immunocytochemistry (Cataldo et al., 1990) using a polyclonal antibody directed against myosin Va. Several sections were processed in tandem in the absence of primary antibody and served as controls.

Immunoelectron microscopy and morphometry of optic and sciatic nerves

3–4-wk-old *dl* and their control mice and NF-L–null mice were anesthetized and perfused fixed with 4% paraformaldehyde–0.2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The tissues were fixed for an additional 2 h at room temperature in 10% formalin. The brain and optic pathway were removed and 40-μm-thick vibratome sections of the cerebral peduncle and the optic pathway at a distance of 700 μm from the retina were dehydrated in series of alcohols and embedded in Epon as previously

described (DeMey, 1983). Ultrathin sections were placed on nickel-coated grids and processed for postembedding immunostaining (DeMey, 1983) using a polyclonal antibody directed against myosin Va. Grids incubated without primary antibody served as controls. All grids were poststained in uranyl acetate and lead citrate and inspected using a JEOL 100EX electron microscope.

Sciatic nerves were dissected and analyzed morphometrically to determine axonal cross-sectional areas for the entire fiber population and neurofilament numbers for a representative subpopulation of axons, as previously described (Rao et al., 1998).

Blot overlay binding assay

Neurofilament triplet subunits were purified as previously reported (Balin et al., 1991). The myosin Va fusion protein (~120 kD) (Engle and Kennett, 1994) consists of a small 3.5-kD piece of β-galactosidase fused to that portion of myosin Va representing 57% of the full-length protein containing half of the actin binding site, calmodulin binding domains, α helical coiled-coil domains, and a small portion of the COOH-terminal tail domain. The fusion protein was expressed in XL-1 Blue *Escherichia coli* (Stratagene) cells and purified using an anti–β-galactosidase affinity column (Promega) per the manufacturer's protocol. 10 μg of purified neurofilament triplet proteins were run per lane on a 7% SDS–polyacrylamide gel, blotted onto nitrocellulose, and cut into strips. The strips were blocked and incubated in 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, or, as a control, 0 μg/ml of myosin Va fusion protein in PBS. Bound myosin Va–β-gal was detected with anti–β-galactosidase mAb (Boehringer) according to the manufacturer's instructions and blots were processed with the ECL system (Amersham Biosciences) and by exposure to Kodak XAR film. The purified neurofilament triplet proteins immobilized on the nitrocellulose were visualized by Coomassie blue staining. Cytoskeletal blot overlay assays were performed same as indicated above for purified NF proteins.

Coimmunoprecipitation of myosin Va and NF-L fusion proteins

Myosin Va–β-gal fusion protein was expressed as described above and the crude extract was used for immunoprecipitation. NF-L myc-tagged plasmid (Heins et al., 1993) was transfected into BL21-DE3 host cells, induced, NF-L–containing lysates were made, and protein concentrations determined. 2 μg of NF-L–FP crude extract and myosin V–FP crude extract (containing ~3 μg of myosin Va–FP) were mixed together in 1× RIPA buffer. Protein A/G–agarose (Boehringer) was also added to preclear. The mixture was incubated overnight at 4°C to allow binding between NF-L–FP and myosin Va–FP. As controls, 2 μg of NF-L–FP or 3 μg of myosin Va–FP, in 1× RIPA buffer were also precleared with protein A/G–agarose overnight at 4°C. Protein A/G–agarose was removed with centrifugation, and NF-L–FP/myosin Va–FP complex, NF-L–FP alone, and myosin Va–FP alone were immunoprecipitated for 8 h at 4°C with either 5 μl of a polyclonal Myc antisera (to precipitate NF-L–FP) or with 2.5 μl of 1:300 dilution of anti–β-galactosidase mAb (Promega). Protein G–agarose (50 μl) was added and the mixtures were nutated overnight at 4°C. Protein G–agarose was pelleted, washed, boiled in Laemmli buffer, fractionated on 7.5% SDS–polyacrylamide gels, blotted onto nitrocellulose membranes, and probed with a mixture of anti–β-gal and anti-Myc antisera.

Immunoprecipitation of myosin Va with NF-L antibody

Triton X-100–insoluble fractions were suspended in TBS, and SDS was added to a final concentration of 1%. The samples were diluted 1:4 in neurofilament extraction buffer (60 mM Tris-HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100, 1 mM PMSF). The samples were sonicated for 20 s and protein was estimated by bicinchoninic acid (BCA) method. A monoclonal antibody to NF-L (NR-4) was added to 0.6–2.4 mg of cytoskeletal fractions at a dilution of 1:10 and the samples were incubated overnight at 4°C. The antigen antibody complex was precipitated using protein A/G–Sepharose (Santa Cruz Biotechnology, Inc.), washed, boiled in Laemmli buffer, fractionated on 7% SDS gels, and immunoblotted with myosin Va and NF-L antibodies.

Immunoprecipitation of ^{35}S-labeled myosin Va

NF-H–null mice were injected intravitreally with 100 μCi of [^{35}S]methionine into each eye and killed after 3 d, as previously described (Nixon and Logvinenko, 1986). Cytosolic and cytoskeletal fractions of optic nerves and tracts obtained were used to immunoprecipitate labeled myosin Va using an affinity-purified polyclonal antibody to myosin Va (Evans et al., 1997). The samples were incubated overnight at 4°C. The antigen–antibody complex was precipitated using protein A/G–Sepharose, electrophoresed, electroblotted, and exposed to X-ray film followed by Western blotting for myosin Va, NF-L, and actin.

Slow axonal transport of pulse-labeled myosin Va and neurofilaments in optic axons

The retinal ganglion cells of 3–4-mo-old NF-H and NF-H/NF-L double null mice were radiolabeled in vivo with 100 μCi of [^{35}S]methionine by intravitreal injection. 3 and 7 d after injection, optic pathways from groups of three animals were cut into eight consecutive 1.1-mm segments. Triton-soluble and -insoluble NF-rich cytoskeleton preparations from each segment were subjected to SDS-PAGE, electrotransfer of proteins, phosphorimaging, and autoradiography.

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References

Balin, B.J., E.A. Clark, J.Q. Trojanowski, and V.M.-Y. Lee. 1991. Neurofilament reassembly in vitro: biochemical, morphological and immuno-electron microscopic studies employing monoclonal antibodies to defined epitopes. *Brain Res.* 556:181–195.

Berg, J.S., B.C. Powell, and R.E. Cheney. 2001. A millennial myosin census. *Mol. Biol. Cell.* 12:780–794.

Bobola, N., R.P. Jansen, T.H. Shin, and K. Nasmyth. 1996. Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells. *Cell.* 84:699–709.

Bridgman, P.C. 1999. Myosin Va movements in normal and *dilute-lethal* axons provide support for a dual filament motor complex. *J. Cell Biol.* 146:1045–1060.

Cataldo, A.M., C.Y. Thayer, E.D. Bird, T.R. Wheelock, and R.A. Nixon. 1990. Lysosomal proteinase antigens are prominently localized within senile plaques of Alzheimer's disease: evidence for a neuronal origin. *Brain Res.* 513(Pt. 2):181–192.

Cheney, R.E., M.K. O'Shea, J.E. Heuser, M.V. Coelho, J.S. Wolenski, E.M. Espreafico, P. Forscher, R.E. Larson, and M.S. Mooseker. 1993. Brain myosin-V is a two-headed unconventional myosin with motor activity. *Cell.* 75: 13–23.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* 16:10881–10890.

DeMey, J.R. 1983. The preparation of immunoglobulin gold conjugates (IGS reagents) and their use as markers for light and electron microscopic immunocytochemistry. *In* Immunocytochemistry. A.C. Cuello, editor. John Wiley & Sons Inc., New York. 358–366.

Engle, L.J., and R.H. Kennett. 1994. Cloning, analysis, and chromosomal localization of myoxin (*MHY12*), the human homologue to the mouse *dilute* gene. *Genomics.* 19:407–416.

Espindola, F.S., E.M. Espreafico, M.V. Coelho, A.R. Marins, F.R.C. Costa, M.S. Mooseker, and R.E. Larson. 1992. Biochemical and immunological characterization of p190–calmodulin complex from vertebrate brain: a novel calmodulin-binding myosin. *J. Cell Biol.* 118:359–368.

Espreafico, E.M., R.E. Cheney, M. Matteoli, A.A.C. Nascimento, P.V.D. Camilli, R.E. Larson, and M.S. Mooseker. 1992. Primary structure and cellular localization of chicken brain myosin V (p190), an unconventional myosin with calmodulin light chains. *J. Cell Biol.* 119:1541–1557.

Evans, L.L., J. Hammer, and P.C. Bridgman. 1997. Subcellular localization of myosin V in nerve growth cones and outgrowth from *dilute-lethal* neurons. *J. Cell Sci.* 110:439–449.

Frappier, T., F. Stetzdowski-Marden, and A. Pradel. 1991. Interaction domains of neurofilament light chain and brain spectrin. *Biochem. J.* 275:521–527.

Hasson, T., P.G. Gillespie, J.A. Garcia, R.B. MacDonald, Y. Zhao, A.G. Yee, M.S. Mooseker, and D.P. Corey. 1997. Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* 137:1287–1307.

Heins, S., P.C. Wong, S. Muller, K. Golide, D.W. Cleveland, and U. Aebi. 1993.

The rod domain of NF-L determines neurofilament architecture, whereas the end domains specify filament assembly and network formation. *J. Cell Biol.* 123:1517–1533.

Hirokawa, N., R. Sato-Yoshitake, N. Kobayashi, K.K. Pfister, G.S. Bloom, and S.T. Brady. 1991. Kinesin associates with anterogradely transported membranous organelles in vivo. *J. Cell Biol.* 114:295–302.

Hirokawa, N., S. Terada, T. Funmakoshi, and S. Terada. 1997. The slow axonal transport: the subunit model. *Trends Cell Biol.* 7:384–388.

Huang, J.-D., S.T. Brady, B.W. Richards, D. Stenoien, J.H. Resau, N.G. Copeland, and N.A. Jenkins. 1999. Direct interaction of microtubule-and-actin based transport motors. *Nature.* 397:267–270.

Jones, J.M., J.-D. Huang, V. Mermall, B.A. Hamilton, M.S. Mooseker, A. Escayg, N.G. Copeland, N.G. Jenkins, and M.H. Meisler. 2000. The mouse neurological mutant flailer expresses a novel hybrid gene derived by exon shuffling between *Gnb5* and *Myo5a. Hum. Mol. Genet.* 9:821–828.

Kayalar, C., T. Ord, M.P. Testa, L.T. Zhong, and D.E. Bredesen. 1996. Cleavage of actin by interleukin 1 β-converting enzyme to reverse DNase I inhibition. *Proc. Natl. Acad. Sci. USA.* 93:2234–2238.

Kim, O.-J., M.A. Ariano, R.A. Lazzarini, M.S. Levine, and D.R. Sibley. 2002. Neurofilament-M interacts with the D_1 dopamine receptor to regulate cell surface expression and densensitization. *J. Neurosci.* 22:5920–5930.

Kobayashi, T., S. Tsukita, S. Tsukita, Y. Yamamoto, and G. Matsumoto. 1986. Subaxolemmal cytoskeleton in squid giant axon. I. Biochemical analysis of microtubules, microfilaments, and their associated high molecular weight proteins. *J. Cell Biol.* 102:1699–1709.

Lewis, S., and R.A. Nixon. 1988. Multiple phosphorylated variants of the high molecular mass subunit of neurofilaments in axons of retinal cell neurons: characterization and evidence for their differential association with stationary and moving neurofilaments. *J. Cell Biol.* 107:2689–2701.

Menasche, G., E. Pastural, J. Feldmann, S. Certain, F. Ersoyy, S. Dupuis, N. Wulffraat, D. Bianchi, A. Fischer, F. Le Deist, and G. de Saint Basile. 2000. Mutations in *RAB27A* cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat. Genet.* 25:173–176.

Mercer, J.A., P.K. Seperack, M.C. Strobel, N.G. Copeland, and N.A. Jenkins. 1991. Novel myosin heavy chain encoded by murine *dilute* coat colour locus. *Nature.* 349:709–713.

Mercken, M., I. Fischer, K.S. Kosik, and R.A. Nixon. 1995. Three distinct axonal transport rates for tau, tubulin, and other microtubule-associated proteins: evidence for dynamic interactions of tau with microtubules in vivo. *J. Neurosci.* 15:8259–8267.

Nixon, R.A. 1991. Axonal transport of cytoskeletal proteins. *In* The Neuronal Cytoskeleton. R.D. Burgoyne, editor. Wiley-Liss, Inc. New York. 283–307.

Nixon, R.A. 1998. Dynamic behavior and organization of cytoskeletal proteins in neurons: reconciling old and new findings. *Bioessays.* 20:798–807.

Nixon, R.A., and K.B. Logvinenko. 1986. Multiple fates of newly synthesized neurofilament proteins: Evidence for a stationary neurofilament network distributed nonuniformly along axons of retinal ganglion cell neurons. *J. Cell Biol.* 102:647–659.

Nixon, R.A., and T.B. Shea. 1992. The dynamics of neuronal intermediate filaments: A developmental perspective. *Cell Motil. Cytoskeleton.* 22:81–91.

Nixon, R.A., I. Fischer, and S.E. Lewis. 1990. Synthesis, axonal transport, and turnover of the high molecular weight microtubule-associated protein MAP 1A in mouse retinal ganglion cells: tubulin and MAP 1A display distinct transport kinetics. *J. Cell Biol.* 110:437–448.

Pastural, E., F.J. Barrat, R. Dufourcq-Lagelouse, S. Certain, O. Sanal, N. Jabado, C. Griscelli, A. Fischer, and G. de Saint Basile. 1997. Griscelli disease maps to chromosome 15q21 and is associated with mutations in the myosin-Va gene. *Nat. Genet.* 16:289–292.

Rao, M.V., M.K. Houseweart, T.L. Williamson, T.O. Crawford, J. Folmer, and D.W. Cleveland. 1998. Neurofilament dependent radial growth of motor axons and axonal organization of neurofilaments does not require the neurofilament heavy subunit (NF-H) or its phosphorylation. *J. Cell Biol.* 143: 171–181.

Rodriguez, O.C., and R.E. Cheney. 2002. Human myosin-Vc is a novel class V myosin expressed in epithelial cells. *J. Cell Sci.* 115(Pt. 5):991–1004.

Sanchez, I., L. Hassinger, P.A. Paskevich, H.D. Shine, and R.A. Nixon. 1996. Oligodendroglia regulate the regional expansion of axon caliber and local accumulation of neurofilaments during development independently of myelin formation. *J. Neurosci.* 16:5095–5105.

Santos, B., and M. Snyder. 1997. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J. Cell Biol.* 136:95–110.

Schnapp, B.J., and T.S. Reese. 1989. Dynein is the motor for retrograde axonal transport of organelles. *Proc. Natl. Acad. Sci. USA.* 86:1548–1552.

Searle, A.G. 1952. A lethal allele of *dilute* in the house mouse. *Heredity.* 6:395–401.

Svitkina, T.M., A.B. Verkhovsky, and G.G. Borisy. 1996. Plectin side arms mediate interaction of intermediate filaments with microtubules and other components of cytoskeleton. *J. Cell Biol.* 135:991–1007.

Tabb, J.S., B.J. Molyneaux, D.L. Cohen, S.A. Kuznetsov, and G.M. Langford. 1998. Transport of ER vesicles on actin filaments in neurons by myosin V. *J. Cell Sci.* 111:3221–3234.

Terry-Lorenzo, R.T., M. Inoue, J.H. Connor, T.A. Haystead, B.N. Armbruster, R.P. Gupta, C.J. Oliver, and S. Shenolikar. 2000. Neurofilament-L is a protein phosphatase-1-binding protein associated with neuronal plasma membrane and post-synaptic density. *J. Biol. Chem.* 275:2439–2446.

Wang, F.S., and D.G. Jay. 1997. Chromophore-assisted laser inactivation (CALI): probing protein function in situ with a high degree of spatial and temporal resolution. *Trends Cell Biol.* 6:442–445.

Wang, L., C.-I. Ho, D. Sun, R.K.H. Liem, and A. Brown. 2000. Rapid movement of axonal neurofilaments interrupted by prolonged pauses. *Nat. Cell Biol.* 2:137–141.

Willard, M. 1977. The identification of two intra-axonally transported polypeptides resembling myosin in some respects in the rabbit visual system. *J. Cell Biol.* 75:1–11.

Wu, Z., K. Rao, M.B. Bowers, N.G. Copeland, N.A. Jenkins, and J.A. Hammer. 2001. Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin to the organelle. *J. Cell Sci.* 114:1091–1100.

Xu, Z., L.C. Cork, J.W. Griffin, and D.W. Cleveland. 1993. Increased expression of neurofilament subunit L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell.* 73:23–33.

Yabe, J.T., W.K. Chang, H.T.M. Chylinski, S. Lee, A.F. Pimenta, and T.B. Shea. 2001. The predominant form in which neurofilament subunits undergo axonal transport varies during axonal initiation, elongation, and maturation. *Cell Motil. Cytoskeleton.* 48:61–83.

Yang, Y., J. Dowling, Q.C. Yu, P. Kouklis, D.W. Cleveland, and E. Fuchs. 1996. An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments. *Cell.* 86:655–665.

Yuan, A., R.G. Mills, C.P. Chia, and J.J. Bray. 2000. Tubulin and neurofilament proteins are transported differently in axons of chicken motoneurons. *Cell. Mol. Neurobiol.* 20:623–632.

Zhu, Q., S. Couillard-Depres, and J.P. Julien. 1997. Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. *Exp. Neurol.* 148: 299–316.