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

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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
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; Esprefico 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 leads to premature death (Pastural et al., 1997). A similar phenotype is associated with mutations of the ashen locus harboring the dilute gene in mice (Dilute/as) and the dilute-lethal (dl) mouse, suggesting that myosin Va plays a role in the behavior of 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 kDa (NF-H), 150 kDa (NF-M), and 70 kDa (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 antiserum against the carboxy-terminal end of myosin Va (Evans et al., 1997) detected a single

![Figure 1](https://example.com/figure1.png)

**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 antibodies against 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.
Myosin Va associates with the NF-L subunit of neurofilaments

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 I. Relative levels of myosin Va in neuronal tissues

| Tissue       | 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.

Myosin Va colocalizes with neurofilaments in axons

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

| Tissue       | 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).
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 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.

**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 αl 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.
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...
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 intravitreal injection of [35S]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 immunoprecipitated with only a limited set of labeled proteins, which included NF-L, 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 intravitreally with [35S]methionine. After 3 or 7 d, the optic pathways
expected 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 [35S]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/μm²) (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 βIII-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
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 (Espreiocco 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 negliglbe 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 Lopovonenko, 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 crossing 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 if 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 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 (DM1A), 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 of the cerebral cortex, and somatic nerves 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 published.
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Slow axonal transport of pulse-labeled myosin Va and neurofilaments in optic axons

The retinal ganglion cells of 3–4-μ-old NF-H and NF-H/NF-L double null mice were radiolabeled in vivo with 100 μCi of [35S]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-electro-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.

Bolada, N., R.P. Jansen, T.H. Shin, and K. Naumysh. 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. Forcier, 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 (IgG agents) and their use as markers for light and electron microscopic immuno-cytochemistry. In Immuno-cytochemistry. A.C. Cuellar, editor. John Wiley & Sons Inc., New York. 358–366.

Engle, L.J., and R.H. Kennett. 1994. Cloning, analysis, and chromosomal localization of myoxin (MYH12) in 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. Manetti, 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. Bridge. 1997. Subcellular localization of myosin V in nerve growth cones and boutons from dilute-lethal neurons. J. Cell Sci. 110:439–449.

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

Hasson, T., P.G. Gilliepe, 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. Golde, 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, O.-J. Kim, M.A. Ariano, R.A. Lazzarini, M.S. Levine, and D.R. Sibley. 2002. 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.

Kim, O.-J., M.A. Ariano, R.A. Lazzarini, M.S. Levine, and D.R. Sibley. 2002. Neurofilament-M interacts with the D, 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. Pastorj, J. Feldmann, S. Certain, F. Ersouy, S. Dupuis, N. Wullfztra, B. 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 genomic 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.* 100:2689–2701.

Meneghini, G., E. Pastorj, J. Feldmann, S. Certain, F. Ersouy, S. Dupuis, N. Wolfluhr, B. 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.

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, 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.

Yang, Y., J. Dowling, Q.C. Yu, P. Koulkis, D.W. Cleveland, and E. Fuchs. 1996. An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments. *Cell.* 86:555–565.

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.