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The Light Chain Composition of Chicken Brain Myosin-Va: Calmodulin, Myosin-II Essential Light Chains, and 8-kDa Dynein Light Chain/PIN

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Class V myosins are a ubiquitously expressed family of actin-based molecular motors. Biochemical studies on myosin-Va from chick brain indicate that this myosin is a two-headed motor with multiple calmodulin light chains associated with the regulatory or neck domain of each heavy chain, a feature consistent with the regulatory effects of Ca^{2+} on this myosin. In this study, the identity of three additional low molecular weight proteins of 23-,17-, and 10 kDa associated with myosin-Va is established. The 23- and 17-kDa subunits are both members of the myosin-II essential light chain gene family, encoded by the chicken L23 and L17 light chain genes, respectively. The 10-kDa subunit is a protein originally identified as a light chain (DLC8) of flagellar and axonemal dynein. The 10-kDa subunit is associated with the tail domain of myosin-Va. Cell Motil. Cytoskeleton 47:269–281, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

The myosin superfamily of actin-based molecular motors consists of at least fourteen structurally distinct classes of unconventional myosins in addition to the well-characterized conventional myosins-II of muscle and nonmuscle cells [Mermall et al., 1998; Wu et al., 2000]. The defining feature of the heavy chain of all myosins is the presence of a structurally conserved N-terminal head or motor domain and, with one known exception [Heintzelman and Schwartzman, 1997], a neck (or regulatory) domain that is followed by a tail domain of varied structure and size, depending on the myosin class. The neck domain is of variable length and consists of one or more IQ motifs, a ~ 24 amino acid segment, each of which serves as a binding site for a myosin light chain [Houdusse et al., 1996; Mooseker and Cheney 1995]. All known myosin light chains are members of the EF-hand superfamily of proteins. These include the family of genes encoding the essential and regulatory light chains associated with the two IQ motifs that comprise the neck domain of myosins-II. *Acanthamoeba* myosin-IC contains a novel EF-hand protein structurally

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more similar to the Ca^{2+} binding protein, calmodulin, than either essential or regulatory light chains [Wang et al., 1997]. Most of the unconventional myosins characterized biochemically thus far utilize one or more copies of calmodulin as light chain [Mooseker and Cheney, 1995]. Recently, however, it was shown that the yeast class V myosin, myo2p, contains both calmodulin light chains and a novel E-F hand light chain termed Mlc1p [Stevens and Davis, 1998]. These neck-associated light chains are thought to be involved in motor regulation as well as maintaining the structural integrity of the helical neck domain, which connects the head and tail domain [Houdusse et al., 1996; Lowey and Trybus, 1995; Rayment et al., 1993; Ruppel and Spudich, 1996]. In conjunction with motor regulation, it has been proposed that the neck, with its associated light chains, may serve as a lever arm of defined length for force transduction, thus defining step size as myosin moves along the actin filament during each cycle of ATP hydrolysis [Highsmith, 1999; Howard, 1997; Huxley, 1998; Uyeda et al., 1996].

Myosin-Va is among the best characterized calmodulin-containing myosins [reviewed in Reck-Peterson et al., 2000]. The heavy chain of myosin-Va is encoded by one of three known vertebrate class V heavy chain genes [Mercer et al., 1991; Rodriguez and Cheney, 1998; Zhao et al., 1996]. In mouse, myosin-Va is encoded by the *dilute* gene [Mercer et al., 1991) and the availability of mutant *dilute* alleles has facilitated functional analysis. Myosin-Va functions revealed from such analyses implicate this myosin in the transport and/or tethering of several classes of organelles whose primary mode of movement is driven by microtubule motors. This includes a role in transport and/or retention of melanosomes within the dendritic (microtubule-free) processes of melanocytes [Lambert et al., 1998; Nascimento et al., 1997; Provance et al., 1996; Wei et al., 1997; Wu et al., 1997, 1998], transport and/or positioning of smooth endoplasmic reticulum within the dendritic (microtubule-free) spines of Purkinje cells [Dekker-Ohno et al., 1996; Takagishi et al., 1996] and the augmentation of microtubule based organelle transport within the axon [Bridgman, 1999] . The recent demonstration that chicken myosin-Va, like the microtubule motor kinesin, is processive, is consistent with the role of myosin-Va in organelle movement [Mehta et al., 1999].

Myosin-Va purified from chick brain has been extensively characterized biochemically. It is a two-headed myosin that exhibits robust actin-activated MgATPase and motor activity that are both regulated by Ca^{2+}, presumably via its calmodulin light chains [Cheney et al., 1993; Nascimento et al., 1996]. These calmodulin light chains may also provide structural support for the neck domain, which consists of six IQ motifs and is $\sim$ 20 nm in length [Cheney et al., 1993]. The coordinated action of myosin-Va's two heads, through lever arm rotation of each neck, may produces the large, processive $\sim$36-nm steps that have been measured for this myosin [Mehta et al., 1999]. However, analysis of purified chick brain myosin-Va revealed the presence of $\sim$ 4–5 calmodulin/heavy chain, not the 6 predicted from its neck structure [Cheney et al., 1993]. This suggested partial loss of calmodulin might occur during purification and, as a consequence, the observed enzymatic and motor activities may not be entirely reflective of the native molecule. Alternatively, two additional subunits of 17- and 23-kDa co-purify with myosin-Va [Cheney et al., 1993] that might occupy IQ sites on the neck domain. In the course of the present study, we also discovered the presence of a third associated protein of $\sim$ 10 kDa. These additional associated proteins may play critical roles in the structure and function of myosin-Va. Given that current [De La Cruz et al., 1999; Trybus et al., 1999; Wang et al., 2000] and future efforts to provide detailed biophysical and structural characterization of myosin-Va require protein quantities obtainable only by in vitro expression, identification and characterization of the association state of these associated proteins with myosin-Va heavy chain is critical.

In the present study, the identity of the 17-, 23-, and 10-kDa light chains of myosin-Va was determined through peptide sequence and molecular weight analysis. The 17- and 23-kDa proteins are members of the essential light chain family that in class II myosins are bound to the first of the two IQ motifs within the neck domain. Sequence analysis identifies the 10-kDa subunit as a protein that interacts with a diverse array of target proteins. This protein was first characterized as one of the light chains (DLC8) of axonemal and cytoplasmic dynein [King et al., 1996; King and Patel-King, 1995] and subsequently as a potential inhibitor (termed PIN) of the neuronal form of NO synthase [Jaffrey and Snyder, 1996]. DLC8/PIN has also been shown to interact with the transcriptional regulator, I kappaB alpha [Crepieux et al., 1997] and with the Bcl-2 family member, Bim [Puthalakath et al., 1999]. In the myosin-Va molecule, DLC8/PIN is tightly associated with the tail domain. The identification of these light chains was reported in an earlier preliminary report [Espindola et al., 1996].

MATERIALS AND METHODS

Microsequence Analysis of Chick Brain Myosin-Va Light Chains

Myosin-Va was purified from 2–3-day-old chick brain tissue as described in Cheney [1998]. To assess whether light chain content of myosin-Va changed as a function of age after hatch, one preparation of myosin-Va

was isolated from 9–10-day-old chicks. The ratio of heavy chain to each of the light chains was determined by densitometry of SDS gels containing a serial dilution of known concentrations of myosin-Va, calmodulin, and bacterially expressed *Chlamydomonas* DLC8. To concentrate the 10-,17-, and 23-kDa light chains, and reduce the amount of calmodulin in the preparation, aliquots of purified myosin-Va, in Buffer A (10 mM imidazole, pH 7.2, 75 mM KCl, 2.5 mM $MgCl_2$, 2.0 mM dithiothreitol, 0.1 mM ethylene glycol tetra-acetic acid [EGTA]) were heat denatured by placement in a boiling water bath for 3 min. Most of the calmodulin remains in the supernate, while the heavy chain and non-calmodulin light chains can be concentrated as a precipitate by sedimentation. The resulting pellets were run on Bio-Rad (Richmond, CA) pre-cast 4–20% gels and transferred to sequence-grade Immobilon filters (Millipore, Bedford, MA) for subsequent sequence analysis. Analysis of tryptic peptides obtained from the excised light chain bands was performed either by the W.M Keck Foundation Biotechnology Resource Laboratory, Yale University, or the Worcester Foundation for Biomedical Research following methods described in King et al. [1996]. Molecular weights of intact light chains and tryptic peptides were determined by mass spectrometry (matrix-assisted-laser-desorption-ionization time-of-flight) using the facilities cited above at both the Worcester Foundation for Biomedical Research and Yale University.

Analysis of Head- and Tail Fragments of Myosin-Va Obtained by Calpain Cleavage

Preparations of myosin-Va (0.1–0.2 μM in buffer A with added 2.0 mM $CaCl_2$) were digested with calpain as described in Nascimento et al. [1996], which results in the formation of stable heavy chain fragments of 65- and 80-kDa derived from the head and tail domains, respectively; only the 10-kDa light chain and calmodulin light chains remain intact after digestion. To determine with which domain the 10-kDa light chain is associated, several methods, including actin cosedimentation, immunoprecipitation, ion exchange chromatography, and calmodulin affinity chromatography, were employed to obtain separated head and tail fragments. Actin cosedimentation (in the absence of ATP) was performed as described in Nascimento et al. [1996]. The head fragment and associated calmodulin light chains, but not the 80-kDa tail fragment, cosediment with F-actin. Actin was purified from chicken skeletal muscle by the method of Spudich and Watt [1971]. Immunoprecipitation of the tail domain from unseparated calpain digests of myosin-Va was performed by overnight incubation at 4°C with 20 μg/ml of either anti-globular tail myosin-Va or non-immune rabbit IgG (Sigma, St. Louis, MO) in the presence of Buffer A with added 150 mM NaCl and 0.2% Triton X-100 followed by addition of protein A Sepharose beads (Pharmacia, Piscataway, NJ). The bead suspensions were gently mixed using a rocking platform for 30 min at 4°C. The protein A Sepharose beads were collected by sedimentation in a microfuge, supernates saved for immunoblot analysis, and the bead pellets were washed once with 0.5M NaCl in Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 150 mM NaCl) and then six times in TBS. The washed pellets, together with the initial supernates, were then dissolved in SDS sample buffer and analyzed for 10-kDa light chain and myosin-Va tail fragment content by SDS-PAGE and immunoblot using anti-DLC8 and anti-tail myosin-Va.

Anion exchange chromatography using Q-Sepharose (Pharmacia, Piscataway, NJ) was performed by applying calpain digests of myosin-Va in Buffer A to ~ 1 ml column followed by stepwise elution with buffer A containing 0.15 M NaCl, followed by 0.75 M NaCl. Calmodulin affinity chromatography (Pharmacia) was performed by applying myosin-Va calpain digests to the column in Buffer A plus 2 mM $CaCl_2$, followed by elution with Buffer A plus 5 mM EGTA and then SDS PAGE sample buffer. For all the above fractionations, the presence of the calmodulin and 10-kDa light chains in the various fractions was ascertained either directly by SDS PAGE or by immunoblot using anti-DLC or anti-calmodulin (UBI, Lake Placid, NY). Flagella isolated from *Chlamydomonas* (gift from the laboratory of Joel Rosenbaum, Yale University) were used as a positive control for the DLC8 blots. All immunoblot analysis was performed by the ECL method following the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). For some experiments, retention of the 10-kDa light chain on the immunoblot was maximized by incubating the PVDF membranes (0.2 μm sequence grade Bio-Rad) with 0.2% of Glutaraldehyde (8% EM grade solution, Electron Microscopy Science, Fort Washington, PA) in TBS for 40 min**,** followed by two washes in TBS prior to blocking and antibody incubation using the standard protocol.

Determination of DLC8/PIN Light Chain Association With Mouse Myosin-Va

To assess whether the association of DLC8/PIN with myosin-Va is unique to chicken, myosin-Va was purified from mouse brain homogenates by immunoprecipitation. Mouse brain tissue (0.2–0.3 gm) was homogenized in 5 ml of homogenization buffer [Cheney, 1998] (40 mM Hepes, pH 7.7, 10 mM K-EDTA, 10 mM ATP, 2 mM DTT, and 1 mM AEBSF) using a glass dounce homogenizer. Homogenates were spun at 30,000*g* for 20 min, and Triton X-100 and NaCl were added to the resulting supernatant to final concentrations of 0.1% and 150 mM, respectively. Myosin-Va was immunoprecipi-

tated from this supernate using affinity purified polyclonal antibodies (2 μg/ml) raised against the globular tail domain of chicken myosin-Va and protein A Sepharose beads (50 μl of beads/0.5 ml of extract). Control immunoprecipitations were done in the absence of primary antibody. After incubation for 1 h at 4°, the protein A beads were collected by pelleting through a 1 M sucrose cushion in HSB (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl) and washed sequentially with 1 M NaCl in HSB and low salt wash buffer (10 mM Tris-HCl, pH 7.5). Washed beads and supernates from the immunoprecipitates were dissolved in SDS PAGE sample buffer and assessed for presence of myosin-Va heavy chain and DLC8/PIN by immunoblot analysis as described above.

Assessment of the Association State of the 10-kDa Light Chain With Myosin-Va

To determine the nature of the binding interaction of the 10-kDa light chain with myosin-Va, aliquots of purified myosin-Va were treated with various conditions and bound and free 10-kDa light chains separated by filtration using Microcon 100 filtration chambers (Amicon, Beverly, MA). Conditions included the following: Buffer A with 1.0 mM EGTA, Buffer A with 1 mM $CaCl_2$ and 10 μM mellitin (Sigma, St. Louis, MO), 100 mM Sodium acetate, pH 5.0 (by addition from a 1.0 M stock), 100 mM Sodium carbonate, pH 11.0 (from a 1.0 M stock), 8.0 M urea (made by addition of solid urea to an aliquot of myosin-Va in Buffer A), and Buffer A containing 0.6 M KI (from a 4.0 M stock). The samples were incubated for 10 min at room temperature, spun in the Microcon separators at 3,000*g* for 10 min. The flow-through was removed and the retentate was washed two times by addition of Buffer A. The retentate and initial flow-through samples were then analyzed by SDS PAGE and immunoblot analysis using the anti-DLC antibody.

To assess the effect of DLC8/PIN removal on the motor properties of myosin-Va, preparations of DLC8/PIN stripped myosin-Va were compared to control preparations using the gliding filament in vitro motility assay [Kron and Spudich, 1986]. Over 100 velocity measurements were made for each preparation using the Metamorph (Universal Imaging Corp., West Chester, PA) image processing system.

Immunolocalization Analysis

Dissociated dorsal root ganglion (DRG) neurons from 10-day-old chicken embryos were cultured on laminin-coated coverslips as previously described [Suter et al., 1995]. After 16 h the cells were fixed with 4% paraformaldehyde, 60 mM Pipes, 25 mM Hepes, pH 7.0, 5 mM EGTA, 3% sucrose for 1 h at room temperature (RT). After washing with PBS (0.15 M NaCl, 20 mM sodium phosphate, pH 7.0), permeabilization with 0.1% Triton X-100 in PBS for 10 min, additional PBS washing and blocking with 5% bovine serum albumin, 10% normal goat serum, 0.5% gelatin in PBS for 30 min, the cells were incubated with primary antibodies in 1:5 blocking solution/PBS for 1 h at 37°C. After washing, secondary antibody incubation was for 30 min at RT. The following primary antibodies were used: (all at 5–10 μg/ml) rat anti-myosin-Va head [Suter et al., 2000], rabbit anti-myosin-Va tail [Suter et al., 2000], mouse anti-dynein intermediate chain, IC 74 (Mab 74.1; gift of K. Pfister, U. Va.; [Dillman and Pfister, 1994]), and rabbit-anti-DLC 8. Secondary Texas Red or fluorescein-labeled antibodies were either from Amersham (Arlington Heights, IL) or Jackson Immuno Research (West Grove, PA). Images were obtained using a Diaphot-300 microscope (Nikon Inc, Melville, NY) with a 100× 1.4 N.A. objective, an Image Point CCD camera (Photometrics, Tucson, AZ) linked to a Metamorph image processing system.

RESULTS

17- and 23-kDa Light Chains Of Chick Myosin-Va Are Encoded by the L17 and L23 Myosin-II Essential Light Chain Genes

Analysis of purified chick myosin-Va on SDS gels revealed the presence of two proteins of 17- and 23-kDa in addition to the prominent calmodulin light chain band [Cheney et al., 1993] (Fig. 1A). These proteins comigrate with the 17-kDa essential light chains associated with myosin-II purified from gizzard and the 17- and 23-kDa essential light chains associated with myosin-II purified from chick brain (Fig. 1A). Based on densitometry of such gels, it was previously determined that the myosin-Va heavy chain:calmodulin ratio is 1:4 ± 0.7 while the heavy chain: 17- and 23-kDa light chain ratios (using calmodulin as a dye binding standard) were 1:0.7 and 1:0.3, respectively, for the several preparations used for quantitation [Cheney et al., 1993].

Microsequence analysis of these light chains was performed with the hope of establishing their relatedness to other known proteins. The light chain region of such gels was electrotransfered to Immobilon paper and the calmodulin, 17- and 23-kDa light chains bands were digested with trypsin. Sequence analysis of a pair of peptides from the presumed calmodulin band (see Table I) exactly matched chicken calmodulin [Simmen et al., 1985] confirming that calmodulin is the major light chain (only indirect assays such as heat stability and Ca^{2+} dependent gel shift assays had been performed previously). Analysis of two peptides each from both the 17- and 23-kDa light chains revealed 100% identity to corresponding sequences reported for the 17- and 23-kDa

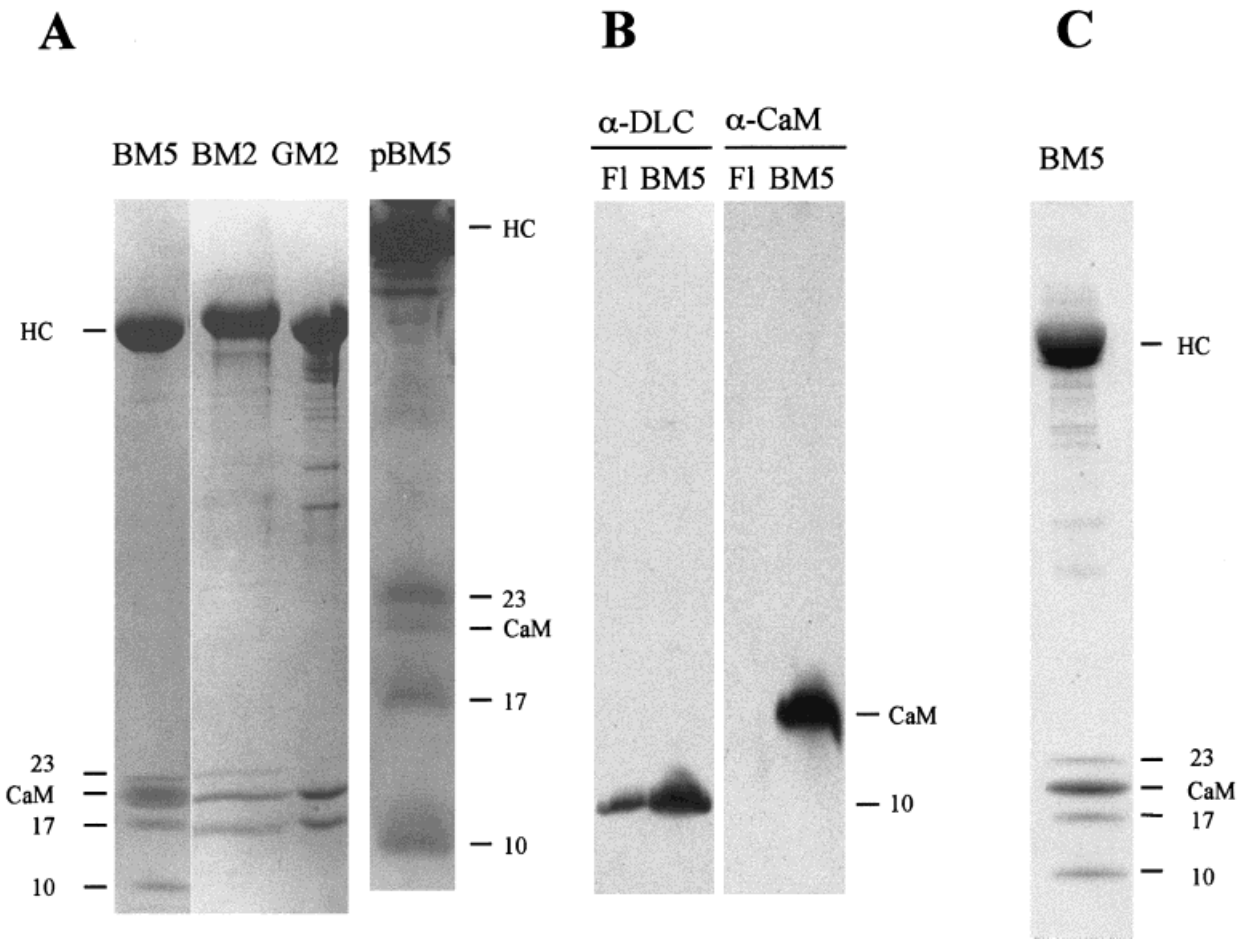


Fig. 1. **A:** Comparison of the light chains of myosin-Va (**lane BM5**; migration position of heavy chain is indicated by HC) with those of myosins-II purified from chick brain (**lane BM2**) and gizzard smooth muscle (**lane GM2**; gift from the laboratory of D.L. Taylor, Carnegie Mellon University) by SDS-PAGE (Coomassie blue staining). The 23 kDa (23) and 17 kDa (17) light chains of myosin-Va co-migrate with the corresponding essential light chain in brain and gizzard myosin II, while the calmodulin (CaM) light chain of myosin-Va co-migrates with the regulatory light chain of these myosins. The migration position of a previously undetected 10 kDa light chain (10) is also indicated. For sequence analysis of light chains, preparations of myosin-Va were concentrated and partially calmodulin depleted by heat denaturation (**lane pBM5**) and run on Bio-Rad 4–20% gels. **B:** Immunoblot analysis of myosin-Va and flagellar axonemes purified from *Chlamydomonas reinhardtii* (Fl) using either anti- *Chlamydomonas* DLC (α-DLC) or anti-calmodulin (α-CaM). **C:** SDS-PAGE analysis of a myosin-Va preparation from 10-day post hatch chicken brain tissue.

essential light chains that are encoded, respectively, by the L17 [Nabeshima et al., 1987] and L23 [Kawashima et al., 1987; Nabeshima et al., 1988] myosin-II essential light chain genes of chicken, both of which are expressed in adult brain (see Table I). Moreover, the molecular weights of five other tryptic peptide peaks for both the 17- and 23-kDa light chains closely matched the molecular weights of peptides predicted from the sequence of these two light chains (Table I).

Based on our preliminary report [Espindola et al., 1996] of the identification of essential light chains in chick myosin-Va, Wang and coworkers [Wang et al., 2000] have recently investigated the presence of essential light chains in myosin-Va purified from adult mouse brain. In contrast to our findings, these workers, using immunoblot analysis, failed to detect essential light chains in their preparation. Since L17 and L23 essential light chain expression in the chicken is developmentally regulated [Kawashima et al., 1987; Nabeshima et al., 1988], one possible reason for the difference in light chain content between chicken and mouse myosin-Va may be that our preparations are routinely prepared from 1–2-day post hatch chicks while the mouse myosin-Va preparation was made from adult brain tissue. To address this issue, a preparation of myosin-Va was prepared from 10-day post-hatch chickens; much of brain development is complete by this time [Rogers, 1995]. Both the 17- and 23-kDa light chains were present in this preparation (Fig. 1C).

TABLE I. Microsequence Analysis of Tryptic Peptides (Shown in Bold) From Myosin-Va Calmodulin, 23- and 17-kDa Light Chains and Comparisons of the Observed Masses of Tryptic Peptides From the 23- and 17-kDa Light Chains With Those Predicted for the L23 and L17 Essential Light Chains*

Light chains	Sequence	Predicted mass (Da)	Observed mass (Da)
CaM	**VFDKDGNGYISAAELR**		ND
CHKCaM (K000430[1,2])	**HVMTNLGEXLTDEEVDEMIR**		ND
LC23	(K) [24]EFTFDPK[30]	884	884
CHKM2 L23 (M34990[3])	(K) [34]IEFAAEQIEEFK[45]	1,455	1,457
	(K) [34]IEFAAEQIEEFKEAFSLFDR[53]	2,420	2,423
	(R) [72]ALGHNPTNAEVLK[84]	1,364	1,365
	(**R**) **[116]EQGTFEDFVEGLR[128]**	1,528	1,528
	(**R**) **[129]VFDKEGNGLVMGAELR**[144]	1,752	1,754
	(R) [145]HVLVTLGEK[153]	996	997
LC17	[1]MCDFSEEQTAEFK[13]	1,535	1,543
CHKM2 L17 (MOCHG2[4,5,6])	(K) [26]ILYSQCGDVMR[37]	1,360	1,360
CHKM2 L17 (MOCH6N[6])	(R) **[37]ALGQNPTNAEVMK[50]**	1,390	1,392
	(K) **[63]TLNFEQFLPMMQTIAK[79]**	1,945	1,947
	(K) [79]NKDQGCFEDYVEGLR[94]	1,845	1,847
	(R) [94]VFDKEGNGTVMGAEIR[110]	1,740	1,743
	(R) [110]HVLVVTLGEK[119]	996	997

*For the presumed CaM and both the 23- and 17-kDa light chains (LC), a pair of tryptic peptides from each light chain was sequenced (**show in bold type**), revealing 100% identity with the corresponding sequences of chicken CaM (CHKCaM) and the 23- and 17-kDa essential light chains encoded by the myosin-II essential light chain genes, L23 and L17 (CHKM2 L23 and L17). [1][Putkey et al., 1983]; [2][Simmen et al., 1985]; [3][Nabeshima et al., 1988]; [4][Matsuda et al., 1981]; [5][Grand and Perry, 1983]; [6][Nabeshima et al., 1987].

TABLE II. Microsequence Analysis Identifies the 10-kDa Light Chain as DLC8/PIN*

Chicken BM5 10 kDa LC	NADMSEDMQQDAVD	DIAAYIK	YNPT
Chlamy DLC (U19490[1])	*[10]*NADMSEEMQADAVD	*[37]*DIAAYIK	*[50]*YNPT
Human (U32944[2])	NADMSEEMQQDSVE	DIAAHIK	YNPT
Bovine brain DLC[3]	NADMSEEMQQDS	DIAAYIK	
Rat PIN (U66461[4])	NADMSEEMQQDSVE	DIAAHIK	YNPT
Mouse PIN (4103059[5])	NADMSEEMQQDSVE	DIAAHIK	YNPT
Rabbit PIN (AF020710[6], 1BKQ[7])	NADMSEEMQQDSVE	DIAAHIK	YNPT
D. melanogaster DYL1 (Q24117[2])	NADMSEEMQQDAVD	DIAAYIK	YNPT

*Sequences of 3 peptides of the myosin-Va (Chicken BM5) 10-kDa lc and corresponding sequences of flagellar and cytoplasmic DLC8 as well as other homologs present in the database from a variety of species are shown. Numbers in italic indicate position in the primary structure. The accession numbers of each sequence, with ref. number are listed. *Chlamy* DLC: *C. reinhardtii* 8 kDa outer arm flagellar dynein light chain; PIN: protein inhibitor of neuronal NO synthase. [1][King and Patel-King, 1995]; [2][Dick et al., 1996]; [3][King et al., 1996]; [4][Jaffrey and Snyder, 1996]; [5]Mount, unpublished data; [6][Jeong et al., 1998];[7] [Tochio et al., 1998].

10-kDa Myosin-Va Light Chain Shares Sequence Identity With the 8-kDa Dynein Light Chain/PIN and Is Present on Both Chicken and Mouse Brain Myosin-Va.

As noted above, SDS-PAGE analysis of purified myosin-Va on 5–20% gels revealed the presence of a third non-calmodulin light chain of ~ 10 kDa that had not been detected on the 5–15% gels used in our earlier studies (Fig. 1A). It is also present in myosin-Va preparations from 10-day post hatch chickens (Fig. 1C). The ratio of heavy chain:10-kDa light chain was estimated by densitometric analysis of Coomassie-blue stained SDS gel lanes containing purified myosin-Va and known amounts of *Chlamydomonas* flagellar DLC8 as standard. A heavy chain:10-kDa light chain ratio of 1:0.97 ± 0.24 was obtained. Results of sequence analysis of 3 tryptic peptides derived from the 10-kDa subunit indicate that these peptides exhibit 85–100% identity to corresponding sequences of a ubiquitously expressed protein first identified as a dynein light chain, DLC8 (actual M_r:10,3 kDa) [King et al., 1996; King and Patel-King, 1995], and subsequently as an interactor with several other proteins including neuronal NO synthase (termed PIN) [Jaffrey and Snyder, 1996], the transcriptional control protein, I

kappaB alpha [Crepieux et al., 1997], and the Bcl-2 family member, Bim [Puthalakath et al., 1999]. Alignment of the 10-kDa peptide sequences with corresponding sequences of DLC8/PIN from a number of species is shown in Table II.

Consistent with the identification of the 10-kDa subunit as DLC8/PIN, antibodies raised against the *Chlamydomonas* DLC8 react strongly with the 10-kDa light chain of myosin-Va (Fig. 1B); see also [Benashski et al., 1997]. Moreover, a sample of myosin-Va subjected to laser desorption mass spectroscopy showed a clear peak with a mass of 10,257 Da, very close to the predicted mass of other vertebrate DLC8/PIN proteins whose sequences have been determined thus far (e.g., 10,365 Da for human DLC8/PIN [Adams et al., 1995]). If the N-terminus is blocked by removal of the N-terminal methionine and acetylation, the expected molecular weight of the human DLC8/PIN would be 10,276 Da.

10-kDa Light Chain Is Associated With the Tail Domain of Myosin-Va

To determine with which myosin-Va heavy chain domain the DLC8/PIN subunit is associated, head and tail fragments of myosin-Va generated by calpain cleavage were analyzed for the presence of this light chain. Limited calpain proteolysis of myosin-Va results in the formation of a stable 80-kDa tail fragment and a 65-kDa head peptide [Nascimento et al., 1996] (see lane dBM5 in Fig. 2). As shown previously [Nascimento et al., 1996], sedimentation of mixtures of F-actin and calpain digests of myosin-Va results in co-pelleting of the 65-kDa head fragment with actin (see lanes 1 and 2, Fig. 2). Note that the calmodulin light chains also cosediment. Since the 65-kDa fragment lacks the neck domain [Nascimento et al., 1996], it is likely that the neck and bound calmodulin light chains remain associated with the head fragment through noncovalent interactions. Unfortunately, both the 23- and 17-kDa essential light chains are cleaved by calpain (compare lanes BM5 and dBM5 in Fig. 2), so their association with the neck domain could not be verified by this method. In contrast to calmodulin, the 80-kDa tail fragment remained in the supernate after cosedimentation (lane 1, Fig. 2). The DLC8/PIN also remains in the supernate fraction (lane 1', Fig. 2).

Several experimental strategies were employed to determine if the 10-kDa subunit is bound to the 80-kDa tail fragment. Immunoprecipitation of the 80-kDa tail fragment using globular tail domain-directed antibodies from calpain digests (Fig. 3) resulted in co-immunoprecipitation of the DLC8/PIN. Second, separation of head and tail fragments was performed using anion exchange chromatography (Fig. 4). The 80-kDa tail domain eluted from such columns at lower salt concentrations than the 65-kDa head fragment and calmodulin (Fig. 4A–C). Immunoblot analysis reveals that the DLC8/PIN co-eluted from this column with the tail fragment (Fig. 4D). Similar separation of head and tail fragments was obtained by both calmodulin affinity and cation exchange chromatography. In both cases, the DLC8/PIN subunit co-eluted with the 80-kDa tail fragment (results not shown).

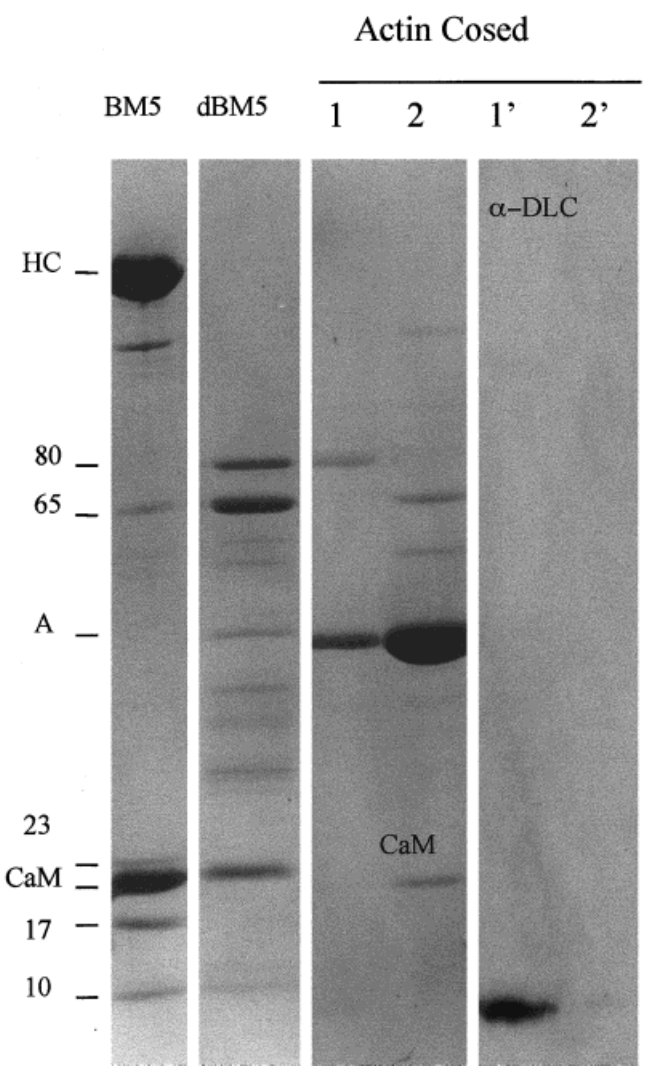
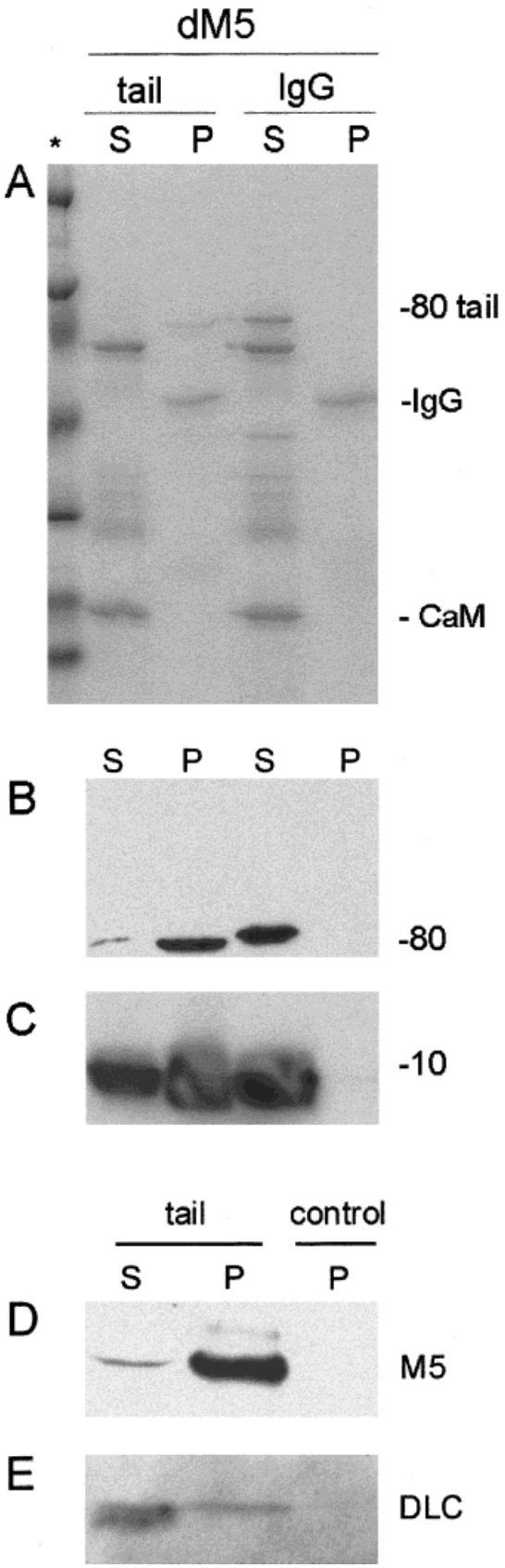


Fig. 2. Separation of calpain-derived head and tail fragments by actin cosedimentation. **Lanes BM5** (intact myosin-Va) and **dBM5** (digested) show protein profiles (Coomassie blue staining of a 4–20% SDS gel) of the myosin-Va preparation before and after calpain digestion. **Lanes 1** and **2** are the supernate (1) and pellet (2) fractions after cosedimentation of the digested myosin-Va with actin. Migration positions of intact myosin-Va heavy chain (HC) the 80-kDa tail- and 65-kDa head fragments, actin (A), and myosin-Va light chains (23, CaM, 17 10) are indicated. **Lanes 1'** and **2'** are immunoblots of the supernate and pellet fractions using anti-DLC8.

Dissociation of the 10-kDa Subunit From Myosin-Va Requires Harsh Solution Conditions

Various solution conditions were tested to assess the association state of DLC8/PIN with myosin-Va



heavy chain (Fig. 5). Bound and free DLC8/PIN were separated by microfiltration using a filtration apparatus with a pore exclusion limit of 100 kDa. Treatment with the calmodulin inhibitor, mellitin, which results in dissociation of the calmodulin light chains, does not release DLC8/PIN. Complete release of DLC8/PIN was achieved in the presence of 0.1 M sodium acetate buffer at pH 5.0 or 0.1M sodium carbonate buffer at pH 11.0. Partial dissociation was effected by treatments with 0.6 M KI and 8.0 M urea. Only acetate treatment yielded selective dissociation of the DLC8/PIN relative to retained calmodulin light chains. Interestingly, such preparations of DLC8/PIN stripped myosin-Va retain motor activity as assessed by the gliding filament assay. Comparable velocities for both acetate-treated (306 nm/s ± SEM 16.2 nm/s) and control preparations (306 nm/s ± SEM 8.7 nm/s) of myosin-Va were observed indicating that the pH 5 acetate treatment does not denature myosin-Va and that the DLC8/PIN is not required for motor activity.

Mouse Myosin-Va Contains DLC8/PIN

To assess whether DLC8/PIN is associated with myosin-Va from other vertebrate species, myosin-Va was isolated by immunoprecipitation from mouse brain homogenates. Immunoblot analysis revealed that DLC8/PIN does co-immunoprecipitate with mouse brain myosin-Va (Fig. 3D,E). Assuming a heavy chain:DLC8/PIN ratio of 1:1, densitometric analysis of quantitative immunoblots of serially diluted supernate and pellet fractions from such immunoprecipitation experiments revealed that only ~ 3% of the total DLC8/PIN present in the brain extract used for immunoprecipitation is associated with myosin-Va.

Fig. 3. Co-immunoprecipitation of the tail domain and DLC8/PIN from calpain digests (dM5) of myosin-Va . **A:** SDS PAGE (Coomassie blue stained) of the supernate (S) and pellet (P) fractions obtained from immunoprecipitation from dM5 using either antibodies to the globular tail domain of myosin-Va (tail) or an identical concentration of non-immune IgG (IgG). The migration positions of the 80-kDa myosin-Va tail fragment (80 tail), IgG, and calmodulin are indicated. **Left lane** contains molecular weight markers. Note that the 80-kDa tail fragment is specifically immunoprecipitated by the anti-myosin-Va tail antibody. The DLC8/PIN subunit is too dilute in these digests to be detected by protein staining. **B,C:** Immunoblot analysis of the supernate and pellet using either anti-globular myosin-Va tail (B) or anti-*Chlamydomonas* DLC8 (C). Note that a significant fraction of the DLC8/PIN present in the digest specifically co-immunoprecipitates with the tail domain of myosin-Va. **D,E:** Immunoblot analysis of immunoprecipitated (IP) myosin-Va from mouse brain extract. The IP pellet and supernate fractions (pellet was loaded 16-fold more concentrated than supernate) were immunoblotted with antibodies to myosin-Va (D) and anti-*Chlamydomonas* DLC (E). Pellets from the control IP are also shown.

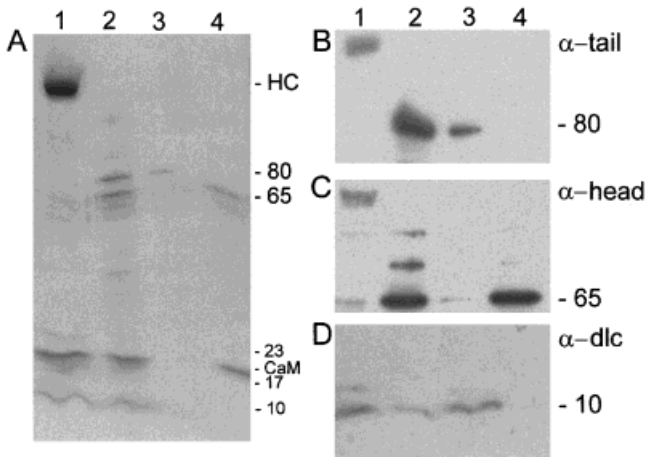


Fig. 4. Separation of myosin-Va head and tail domains by Q-Sepharose chromatography. **A:** SDS-PAGE (Coomassie blue stained) of intact myosin-Va (**lane 1**), the calpain digest (**lane 2**), and fractions eluted from the column by 0.15 M NaCl (**lane 3**) and 0.75 M NaCl (**lane 4**). Migration positions of the myosin-Va heavy chain (HC), 80-kDa tail fragment (80), 65-kDa head fragment (65), calmodulin (CaM), and the 23-,17-, and 10-kDa light chains are indicated (23,17, and 10). **B–D:** Immunoblot analysis of gels identical to that in A, using antibodies to the globular tail (B; α-tail), head (C; α-head) domain, and anti-*Chlamydomonas* DLC (C; α-dlc). Note that DLC8/PIN co-elutes from the column with the 80-kDa calpain-derived tail fragment.

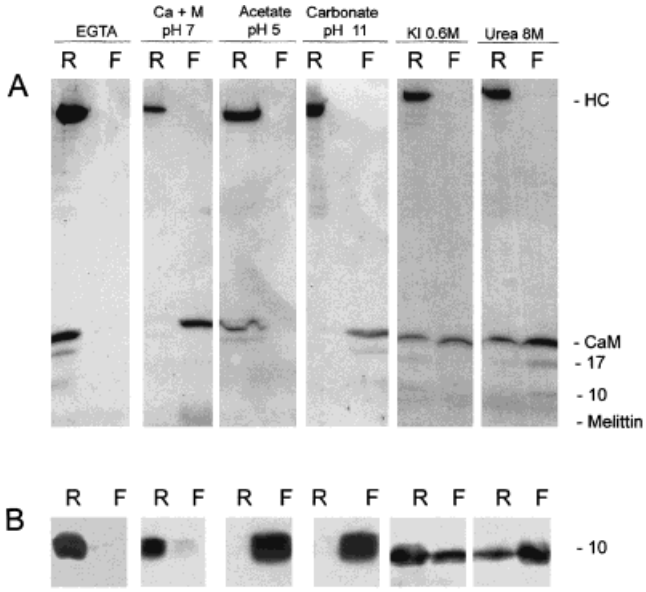


Fig. 5. Dissociation of DLC8/PIN from myosin-Va heavy chain requires harsh solution conditions. **A:** SDS-PAGE (Coomassie stain) of the retained (R) and flow-through (F) fractions of myosin-Va samples subjected to microfiltration after treatment with various solution conditions including control conditions (EGTA), mellitin with 1 mM $CaCl_2$ (Ca + M pH 7), 0.1M sodium acetate at pH 5.0 (Acetate pH 5), 0.1 M sodium carbonate at pH 11 (Carbonate pH 11), 0.6M KI, and 8.0 M urea. Migration positions of myosin-Va heavy chain (HC), calmodulin (CaM), 17-kDa light chain (17), DLC8/PIN (10), and melittin are indicated. Recovery of myosin-Va from the retentate fraction varied due to loss of protein due to adsorption onto the filter membrane under some conditions, the most severe of which was melittin treatment. **B:** Immunoblot analysis of the same retentate (R) and flow-through (F) fractions with anti-*Chlamydomonas* DLC8.

Myosin-Va Partially Co-Distributes With DLC8/PIN But Not Dynein Within the Growth Cones of Cultured Neurons

The localization of myosin-Va, DLC8/PIN, and cytoplasmic dynein was examined in cultured DRG neurons from chick embryos. Dynein localization was determined using an antibody raised against the 74-kDa intermediate chain (DIC 74) of cytoplasmic dynein [Dillman and Pfister, 1994]. All three proteins were distributed throughout these neurons, exhibiting generally punctate staining in cell bodies, neurite shafts, and growth cones (results not shown). With respect to myosin-Va, these results were comparable to those reported previously for both chick and rodent neurons [Espreafico et al., 1992; Bridgman 1999; Suter et al., 2000; Evans et al., 1997]. Because of staining intensity and dense packing of puncta containing myosin-Va elsewhere in these neurons, the peripheral domain of the growth cone was the best region to compare the distribution of myosin-Va with the localization of DLC8/PIN and dynein (Fig. 6). Co-staining with anti-*Chlamydomonas* DLC8 revealed some overlap in distribution with myosin-Va in the growth cone (Fig. 6A–C). However, the coincidence of staining was not complete, as one would expect since the DLC8/PIN interacts with multiple proteins including dynein. DIC 74 staining was qualitatively similar to that of myosin-Va in that within the growth cone, a punctate distribution was observed; however, much less overlap with myosin-Va staining, compared to that with the DLC8 antibody, was seen (Fig. 6D–F). Thus, one potential function for DLC8/PIN, to target both motors to the same cargo vesicles via a common linker complex, is not supported by these observations.

DISCUSSION

The presence of a single essential light chain/heavy chain is a ubiquitous feature of all known class II myosins that have been biochemically characterized. The precise function of the essential light chain is unknown, although its presence is required for myosin-II activities [Lowey and Trybus, 1995; Ruppel and Spudich, 1996]. It seems likely that its association with the most proximal IQ site in the neck domain serves to maintain the structural integrity of this portion of the heavy chain [Lowey and Trybus, 1995; Ruppel and Spudich, 1996; Trybus, 1994]. Presumably, the essential light chains of myosin-Va serve a similar function within the neck domain of this unconventional myosin. However, because calpain

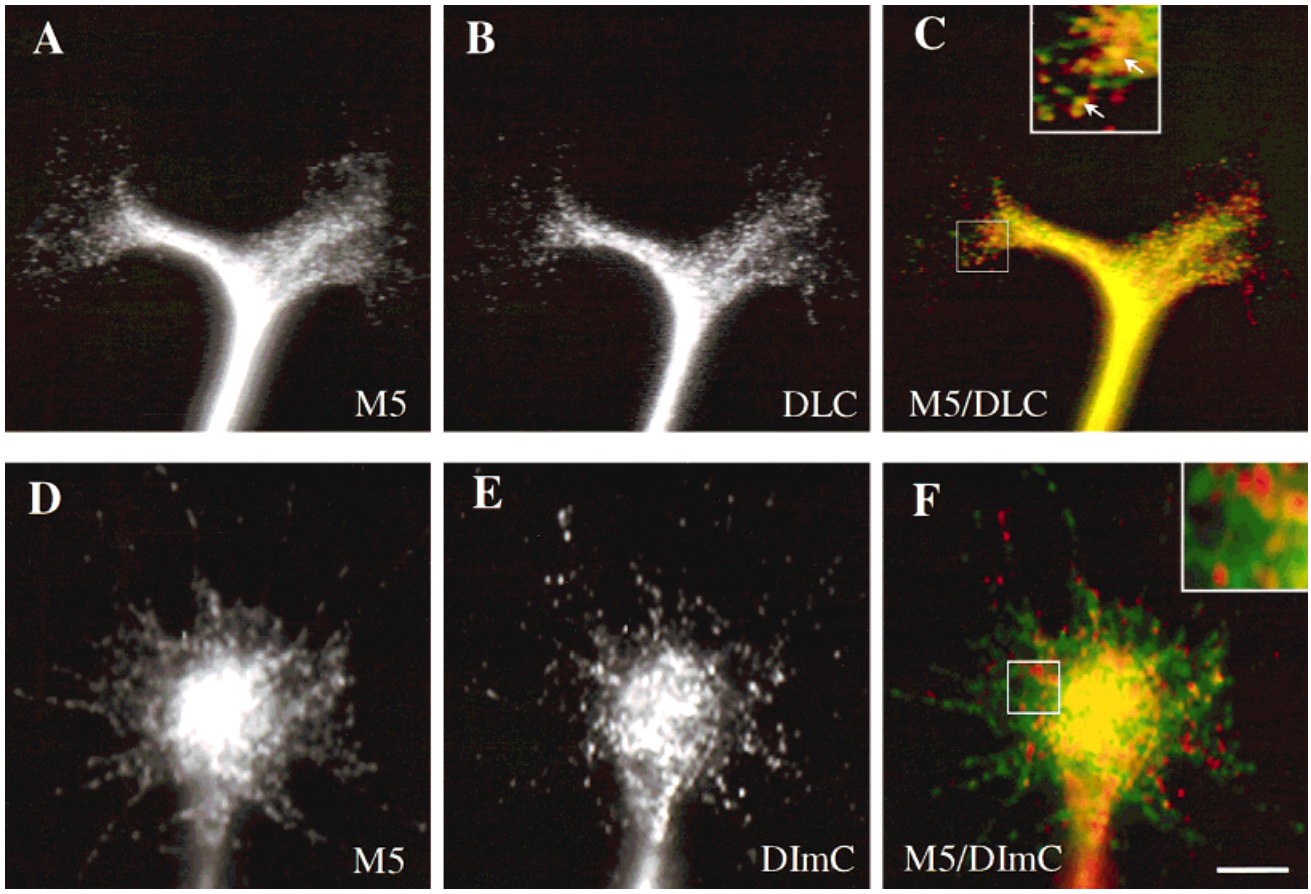


Fig. 6. Localization of myosin-Va, DLC 8, and DIC 74 in growth cones of cultured chick DRG neurons. **A–C:** Dual immunostaining with antibodies to myosin-Va (A) and DLC (B); a pseudocolor overlay of the two images with myosin-Va in green and DLC in red is shown in C. The higher magnification **inset** shows a portion of the peripheral growth cone region, revealing that some of the discrete puncta in this region stain with both myosin-Va and DLC antibodies (*arrows*). **D–F:** Dual immunostaining with antibodies to myosin-Va (D) and DIC 74 (E) and corresponding pseudo-color overlay of the two images with myosin-Va in green and DIC 74 staining in red (F). The higher magnification **inset** in F of a portion of the growth cone peripheral region, where discretely stained puncta can be discerned, reveals little overlap in staining with these two antibodies. Bar = 10 μm; insets are shown at 2.5× the magnification.

digestion results in cleavage of both the 17- and 23-kDa light chains, we can only assume, albeit reasonably, that these light chains are associated with the neck domain. Key unresolved questions concerning the role of the essential light chains include the following: Do the essential light chains of chicken myosin-Va bind to the most proximal IQ site? Recent analysis of in vitro-expressed chicken myosin-Va constructs consisting of the head and first IQ motif bind either calmodulin or essential light chain, and no significant enzymatic differences were reported [De La Cruz et al., 1999]. Do populations of myosin-Va molecules consist of heterodimers or homodimers with respect to the 17- and 23-kDa light chains and is there a single essential light chain/heavy chain? Does calmodulin/essential light chain composition vary in different cell types of the brain or during embryogenesis? In this regard, we have purified myosin-Va from dissected cerebellum and cortex, and observed no difference in light chain content (Espindola and Mooseker, unpublished observations). As noted above, it has recently been demonstrated that mouse myosin-Va lacks essential light chains. We demonstrate that this is unlikely to be due to the difference in developmental age of the brain tissue used for isolation (adult mice vs. 1–2-day-old chickens) since the essential light chains are present in myosin-Va purified from 9–10 day post hatch chickens. This raises the interesting possibility that their differing light chain content may contribute to differences in mechanochemical activity. For example, chicken myosin-Va is a processive motor, while Wang and coworkers report that mouse myosin-Va, while exhibiting very high affinity for actin like chicken myosin-Va [Nascimento et al., 1996], may not be processive [Wang et al., 2000]. However, such differences also

could be contributed by the respective heavy chains, since although highly homologous (motor domains are ~ 94% identical), sequence identities in key regions including the region of actin contact (surface loop 2 in myosin-II) and the first IQ motif, the putative essential light chain binding site, show less identity (83 and 78% identical, respectively).

In considering functions for DLC8/PIN within the myosin-Va molecule, the demonstration of its association with motor proteins may be misleading since as noted previously, this highly conserved, ubiquitously expressed protein may interact with a functionally diverse array of binding partners [Crepieux et al., 1997; Jaffrey and Snyder 1996; Puthalakath et al., 1999]. However, significant insights into how this small protein may interact with such a wide range of target proteins has been provided by recent analyses of DLC8/PIN structure [Fan et al., 1998; Liang et al., 1999] and oligomerization state. Crosslinking studies indicate that DLC8/PIN is a dimer in both dynein and myosin-Va [Benashski et al., 1997]. The structure of the DLC8/PIN dimer, with bound peptides from the PIN binding domain of neuronal NO synthase has recently been solved by X-ray diffraction [Liang et al., 1999]. The monomer consists in a highly polarized molecule, with one face consisting of a pair of alpha helices (solvent facing) and the other a largely hydrophobic beta sheet consisting of 5 beta strands. This sheet constitutes the dimerization domain, and, within the dimer, one of the beta strands in each monomer is contributed by the opposing monomer, presumably stabilizing the dimer. The dimer contains two NO synthase peptide binding clefts at either side of this dimerization domain. From the sequence of the NO synthase peptide that binds to this cleft, a consensus sequence for DLC8/PIN binding has been suggested (Asp-Thr-X-Gln-Val-Asp-X) and similar sequences have been identified in the dynein intermediate chain [Liang et al., 1999]. These authors also incorrectly identify a similar sequence in the tail domain of myosin-Va (the sequence identified is actually from a myosin-II). No similar sequence is present in the myosin-Va heavy chain. Nevertheless, like the target peptide binding domain of calmodulin, these hydrophobic binding clefts might interact with a wide range of peptide segments that lack primary structure similarity. Based on these structural studies, one possible function for DLC8/PIN is to stabilize heavy chain-heavy chain interactions/structure within the globular tail of myosin-Va. Consistent with this idea is the observation that each myosin-Va molecule may contain a single DLC8/PIN dimer, as estimated by gel densitometry. Moreover, electron microscopy of myosin-Va molecules indicates that the distal globular tail domain is generally not bi-lobed, suggesting significant stabilization of chain-chain interactions in this region of the molecule [Cheney et al., 1993]. If the primary function for DLC8/PIN is to link the myosin-Va molecule to other target proteins (e.g., cargo docking components), then one might expect there to be one dimer/heavy chain, not molecule. However, densitometric estimates are just that, and some DLC8/PIN may be lost from myosin-Va during purification.

One intriguing potential "cargo binding " function for DLC8/PIN in myosin-Va is that it may tether the motor to particular mRNA transcripts. This possibility is raised by the recent finding that DLC8/PIN binds to the 3′ UTR of parathyroid hormone mRNA, a transcript that is localized to the microtubule cytoskeleton [Epstein et al., 2000]. Thus, both cytoplasmic dynein and myosin-Va could play a role, via their respective associated DLC8/PIN light chains, in the localization and/or transport of specific mRNAs.

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