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Subcellular localization of GFP-myosin-V in live mouse melanocytes

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SUMMARY

Class-V myosins are two-headed actin-based mechanoenzymes that function in the transport and subcellular localization of organelles and possibly in the outgrowth of cellular processes. To determine which domains of myosin-V are involved in intracellular localization of this motor protein, we have expressed fusions of the green fluorescent protein with segments from two distinct myosin-V heavy chains. The expression patterns of constructs encoding four different domains of chick brain myosin-Va were compared to a single construct encoding the globular tail region of mouse myosin-Vb. In transfected mouse melanocytes, expression of the NH_2-terminal head (catalytic domain) of chick brain myosin-Va codistributed with actin filaments throughout the cytoplasm. A similar construct encoding the myosin–Va head with the associated neck (light chain binding sites), also codistributed with actin filaments. The GFP-head-neck peptide was also highly concentrated in the tips of filopodia in B16 melanocytes wild type for myosin-Va (*MYO5a* gene), but was concentrated throughout the entire filopodia of S91-6 melanocytes derived from *dilute* mice with mutations in the *MYO5a* gene. Evidence is also presented that the globular tail of myosin-Va, but not myosin-Vb, targets this motor molecule to the centrosome as confirmed by colocalization in cells stained with antibodies to γ-tubulin. Expression of the GFP-myosin-Va globular tail causes displacement of endogenous myosin-V from centrosomes as visualized by immunolabeling with antibodies to the head domain of myosin-V. Treatment with the microtubule-disrupting drug nocodazole markedly reduces myosin-V staining at the centrosome. In contrast, there was no detectable diminution of myosin-V staining at the centrosome in cells treated with the actin filament-disrupting drug cytochalasin D. Thus, while localization of the myosin-V motor domain to actin-rich regions is consistent with conventional models of actomyosin-based motility, localization to the centrosome occurs in the complete absence of the myosin-V motor domain and is dependent on intact microtubules.

Key words: Myosin, Myosin-V, Molecular motor, Melanocyte, Green fluorescent protein, Nocodazole, Cytochalasin D

INTRODUCTION

An important issue in understanding possible functions of myosin-V is the elucidation of the molecular domains involved in targeting this motor molecule to specific intracellular sites. For many unconventional myosins, it is commonly assumed that the COOH-terminal tail domain is involved in membrane binding and/or organelle-specific targeting. Thus differences in tail domains may dictate the specific functions thought to be performed by members in the 15 different classes comprising the myosin superfamily (reviewed by Mermall et al., 1998; Probst et al., 1998). Myosin localization and function may also be modulated by factors other than tail domain-associated ligands. For example, several tropomyosins have been demonstrated to differentially regulate the mechanochemistry associated with the head domains of class I, II and V myosins (reviewed by Wolenski, 1995). Hence myosins may localize (or be excluded) at specific intracellular sites due to differential motor activity along tropomyosin-coated actin filament tracks. This possibility is further underscored by the recent results of Pruyne et al. (1998) who demonstrated that tropomyosin-containing actin filaments direct the delivery of vesicles containing Myo-2p (myosin-V) to sites of polarized growth in budding yeast.

Through a combination of biochemical, genetic and immunofluorescence observations, a model has emerged for myosin in the directed transport of vesicles and organelles (reviewed by Mermall et al., 1998). Similar to the microtubule motor kinesin, certain myosins may use their motor domains to walk along actin filaments while pulling specific cargo attached to their tails. The most likely candidates for actin-based organelle transport are class-V myosins (reviewed by Titus, 1997). This class includes the proteins encoded by the yeast *MYO2* (Johnston et al., 1991) and *MYO4* genes (Haarer

et al., 1994), as well as the mouse *MYO5a* (*dilute*) gene product (Mercer et al., 1991), and its avian ortholog chick brain myosin-Va (Espreafico et al., 1992; Sanders et al., 1992), human myosin-Va (*MYH12*, GenBank accession number Y07759), *Drosophila* myosin-V (Bonafe and Sellers, 1998), *Caenorhabditis elegans* hum-2 (Baker and Titus, 1997) and rat myosin-Vb (*myr6*, Zhao et al., 1996). In the yeast Myo2p mutant, *myo2-66*, cell growth and budding are inhibited and vesicles that normally localize with actin filaments to the growing bud accumulate in the mother cell at restrictive temperatures (Johnston et al., 1991; Govindan et al., 1995). Thus Myo2p expression is likely to be essential for vesicle transport along actin filaments to areas of active growth. Myo4p, the gene product of *MYO4*, doesn't appear to be associated with a vesicle population, but this protein is required for the polarized distribution of the protein Ash1p, and probably *ASH1* mRNA, to the growing bud (Bobola et al., 1996; Long et al., 1997; Takizawa et al., 1997).

Informative studies using *dilute* mice, which have mutations in the *MYO5a* gene encoding myosin-Va, corroborate the yeast data and suggest this motor may transport one or more organelles along actin filaments. Mutations at the *dilute* locus result in a 'dilution' of coat color due to the failure of pigment-containing organelles to be properly transported into epidermal keratinocytes at the base of the hair shaft from their site of synthesis in melanocytes (Jackson, 1994; Silvers, 1979). In recent studies it has been demonstrated that cultured melanocytes from *dilute* mice have normal dendritic morphology, but exhibit a concentrated perinuclear distribution of melanosomes (Provance et al., 1996; Wei et al., 1997). Moreover, the outgrowth of superior cervical ganglia neurons from *dilute-lethal* mice appears normal, as do the actin cytoskeleton and filopodia morphology (Evans et al., 1997). Thus the defect in organelle transport in *dilute* mice is likely to be in the outward motility of melanosomes from the perinuclear region to the extensive dendritic arbors of melanocytes (Provance et al., 1996; Wei et al., 1997; Wu et al., 1997, 1998b). Additional support for the role of the *dilute* gene product in melanosome motility and subcellular localization was provided by immunolocalization studies demonstrating that myosin-V colocalizes to melanosomes of wild-type mice melanocytes (Wu et al., 1997; Nascimento et al., 1997) and humans (Lambert et al., 1998).

Nascimento et al. (1997) also demonstrated that myosin-V in melanocytes colocalizes with endoplasmic reticulum, Golgi apparatus and mitochondria, and is concentrated in dendritic tips and at a distinct perinuclear dot. A more detailed immunofluorescence analysis of the perinuclear myosin-V dot revealed that this motor protein is localized to the centrosome (Espreafico et al., 1998). In contrast, Wu et al. (1997) did not observe any striking overlap between myosin-V fluorescence and ER or Golgi, although they did confirm that not all myosin-V was associated with melanosomes. A subsequent immunofluorescence study by Wu et al. (1998a) demonstrated that myosin-Va is associated with microtubule-rich domains in a variety of interphase and dividing cells. Using a combination of immunopurification, coprecipitation and chemical crosslinking assays, Prekeris and Terrian (1997) demonstrated that myosin-V in neuronal tissue forms a stable complex with synaptic vesicles enriched with synaptobrevin II and synaptophysin. Similar findings have been reported by Evans et al. (1998), who found that mechanochemically competent myosin-V is present on brain-derived vesicles containing the synaptic vesicle marker protein SV2.

The best characterized class-V myosin is chick brain myosin-V (BM-V; Cheney et al., 1993; Nascimento et al., 1996). The 212 kDa heavy chain of this myosin shares 91% amino acid identity with the wild-type *dilute* myosin-Va. BM-V is a two-headed mechanoenzyme that has six light chain binding motifs in the neck domain (Espreafico et al., 1992), as well as a site for binding an 8 kDa light chain of cytoplasmic dynein in the myosin tail (Espindola et al., 1996). The proximal region of the elongated tail has coiled-coil α-helical domains responsible for dimerization, but unlike conventional myosin-II in muscle, BM-V does not form bipolar filaments (Cheney et al., 1993). The distal half of the tail has poorly characterized globular domains that appear unable to bind actin (Nascimento et al., 1996). These domains share homology with the *Drosophila* protein *canoe* and human AF-6 (Pointing, 1995). The tail domain of vertebrate myosin-V (Prekeris and Terrian, 1997) and Myo2p (Catlett and Weisman, 1998) may be involved in binding vesicle membrane proteins. BM-V mechanochemistry has been investigated directly using various in vitro motility assays (Wolenski et al., 1993; Cheney et al., 1993) and indirectly using chromatophore-assisted laser inactivation (CALI) in living neurons (Wang et al., 1996). The ability of BM-V to translocate actin filaments is directly regulated by calmodulin light chain binding in a Ca^{2+}-dependent manner (reviewed by Wolenski, 1995). Studies on the enzymology of BM-V indicate that very low concentrations of F-actin can activate the MgATPase (K_{ATPase} 1.3 μM) of this motor (Nascimento et al., 1996). These investigators also demonstrated that, compared to myosin-II, BM-V binds with very high affinity to F-actin in the presence of ATP, particularly in the presence of Ca^{2+} (Nascimento et al., 1996). Hence, only a handful BM-V molecules may need to be associated with organelles for transport over actin tracks.

In this paper, we have used various myosin-V domains fused to green fluorescent protein (GFP) to examine the myosin heavy chain domains involved in intracellular localization and function in transfected mouse melanocytes. The expression of constructs containing domains from avian BM-Va heavy chain were compared to a GFP-construct fused to the tail domain of mouse myosin-Vb and GFP alone. We show that the myosin-Va head and the head-neck domains codistribute with actin filaments throughout the cytoplasm. The head-neck peptide also is differentially concentrated in filopodia in a cell-specific manner. Myosin-Va globular tail fluorescence routinely localized to a distinct perinuclear dot that also stained with antibodies specific for γ-tubulin, reaffirming that the tail domain is responsible for myosin-V association with centrosomes (Espreafico et al., 1998). Analysis of cells treated with either nocodazole or cytochalasin D demonstrated that intact microtubules, but not actin filaments, were necessary for myosin-V localization at the centrosome.

MATERIALS AND METHODS

Cell lines

Murine melanoma cell lines B16-F10 (derived from C57BL/6J mouse, *D/D, B/B*), and S91-6 (isolated from a DBA/2J mouse d^v/d^v, *b/b*) were

acquired as a generous gift from Dr J. Pawelek (Yale University, New Haven, CT). Cells were grown in Ham-F10 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2/95% air atmosphere.

Plasmid constructions

DNA fragments coding for avian brain myosin-Va domains and the globular tail domain of mouse brain myosin-Vb were each inserted into the vector pS65T-C1 (Clontech, Palo Alto, CA), in fusion with the *gfp* open reading frame so that the green fluorescent protein (GFP) was located at the amino terminus of the expressed chimeric proteins (Fig. 1). The sites of ligation were confirmed by DNA sequencing. The following subcloning strategies were used for generation of GFP-myosin-Va constructs. Head: the DNA fragment (nt: 81-2374) corresponding to the entire head domain (aa: 1-764) was extracted from a construct (pCB6-Head-cmyc) previously obtained in the laboratory by PCR and fully sequenced. This construct was digested with *Eco*RI, blunted with Klenow, and digested with *Kpn*I producing the DNA fragment corresponding to the head region that was subcloned into the *Kpn*I and *Sma*I sites of pS65T-C1. Head-neck: A construct containing the sequence encoding the entire head-neck regions plus 13 aa residues of the proximal tail (nt: 81-2847, aa: 1-922) was generated previously in pBS by sequential subcloning from three myosin-Va clones isolated from the cDNA library (N21, H54 H11; Espreafico et al., 1992). This construct was used to generate another construct in the PCB6 mammalian expression vector, pCB-Head-neck-25. This construct was digested with *Hin*dIII, filled in using Klenow, and digested again with *Kpn*I to produce the Head-neck DNA fragment that was subcloned into the *Kpn*I and *Sma*I sites of pS65T-C1. Medial tail: a *Xmn*I fragment of chicken myosin-Va (nt 3424-4386, aa 1115-1435) was extracted from the full-length myosin-Va construct (pCB6-FLMVa) previously obtained and characterized in the laboratory by splicing together clones N21, H54, H11 and 32a. To generate the GFP-medial tail construct, the *Xmn*I DNA fragment was then subcloned into a Klenow-blunted *Xno*I site of pS65T-C1. Globular tail: a DNA fragment corresponding to the globular tail of chicken brain myosin-Va was generated by digestion of the original 32a clone in p-Bluescript with *Pst*I and *Eco*RV (nt 4212-5622, aa 1377-1830) and then subcloned into vector pS56T-C1 as described previously (Espreafico et al., 1998). Myosin-Vb globular tail: the sequence for the globular tail of myosin-Vb was generated by RT-PCR from adult mouse brain RNA, and cloned into the *Bam*HI site of the vector pIH902. This *Bam*HI fragment, including most of the globular tail region, was subcloned into the *Bam*HI site of pS65T-C1. The start of the murine globular tail region in this construct corresponds to amino acid position 1466 of chick brain myosin-Va, based on the sequence alignments of the primary structure of the tail domain of class-V myosins (Espreafico et al., 1992).

Transfection

Cells were grown in the appropriate culture medium, harvested in Tyrode's solution (0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, pH 7.2, 5.5 mM glucose, 137 mM NaCl, 2.7 mM KCl, 0.68 mM EDTA), centrifuged and resuspended in 400 μl of RPMI 1640 culture medium (Gibco-BRL, Gaithersburg, MD) to give approximately 10^7 cells/ml. Cells were transferred to sterile electroporation cuvettes (4 mm gap, Bio-Rad Laboratories Inc, Hercules, CA), mixed with DEAE-Dextran and DMSO at a final concentration of 10 μg/ml and 1.25%, respectively, and 3-6 μg of purified plasmid DNA. The mixture was incubated at room temperature for 15 minutes prior to electroporation. Electroporation was performed in a Bio-Rad GenePulser electroporator at 260 V, 960 μFaradays. Electroporated cells were incubated at room temperature for 10 minutes plated on 22 mm^2 coverslips and incubated under standard conditions.

Treatment with cytoskeletal inhibitors

Cytochalasin D (CD) and nocodazole were purchased from Sigma Chemical Co. and stored as frozen stocks (–20°C) in DMSO. Cultured melanocytes were incubated with either 10 μg/ml CD (to inhibit actin polymerization) or 20 μg/ml nocodazole (to disrupt microtubules) in media at 37°C for up to 24 hours. To inhibit microtubule polymerization completely, some melanocytes were incubated at 4°C for 30 minutes followed by incubation with 10 μg/ml nocodazole. The final concentrations of cytoskeletal inhibitors applied to cells contained 0.2% DMSO. At the indicated times, cells were removed from drug, rinsed twice with warm PBS and fixed as described below.

Immunocytochemistry

Cells were washed with PBS, fixed at 37°C for 30 minutes with 4% paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100 in fixative for 5 minutes followed by washing with PBS. In experiments using nocodazole and cytochalasin D, the fixative was made with 50 mM EGTA in PBS, and permeabilization was performed by submersion in methanol for 10 minutes (–20°C). For staining of filamentous actin, cells were then incubated with rhodamine-phalloidin (1:100, Molecular Probes, Eugene, OR) at room temperature for 20 minutes. For antibody staining, cells were first incubated in 5% bovine serum albumin for 15 minutes, washed with PBS, then incubated in primary antibodies for 45 minutes, washed thoroughly with PBS, incubated in secondary antibodies for 30 minutes, and then washed with PBS. Coverslips were mounted with ProLong Antifade (Molecular Probes) to retard photobleaching.

Antibodies

The rabbit affinity purified polyclonal antibody against *Xenopus* γ-tubulin was a generous gift from Drs R. Palazzo and Andrew Suddith (University of Kansas, Lawrence, KS) and used at a 1:500 dilution. The serum against the myosin-V head domain was a gift from Maria Cristina Ramos Costa (FMRP, USP, São Paulo, Brazil) and used at 4.5 μg/ml. The mouse monoclonal antibody against β-tubulin (1:200) was obtained from Sigma Chemical Co. Secondary antibodies conjugated to either Texas Red (Rockland, Gilbertsville, PA) or rhodamine red (Molecular Probes) were used at 1:500 dilutions.

Imaging

Electroporated cells were grown on coverslips for 48 hours prior to assembly of flow cells (Wolenski et al., 1993) for fluorescence observations. The cells were washed with mouse Ringer's solution (155 mM NaCl, 3 mM KCl, 5 mM Hepes, pH 7.2, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM glucose) and maintained in this solution throughout imaging. An air curtain was used to maintain the stage temperature at 37°C (±2°C). Cells were observed on a Nikon Diaphot 300 inverted microscope equipped with phase contrast and epifluorescence optics (fluorescent filter cubes: Chroma Technology Inc., Brattleboro, VT) using a PlanApo ×63 1.4 NA oil immersion objective (Carl Zeiss). Images were acquired with an Imagepoint cooled CCD camera (Photometrics Ltd, Tucson, AZ) and frame averaged and digitally processed using MetaMorph Image acquisition and processing software (Universal Imaging Corp., West Chester, PA). Confocal microscopy of cells was performed using a Bio-Rad MRC-1024 Krypton/Argon laser scanner mounted to a 2FL reflector slider on a Zeiss Axiovert equipped with DIC optics (PlanApo ×100 1.4 NA oil objective, Carl Zeiss).

RESULTS

Expression constructs

To identify the domains of myosin-V (Fig. 1) involved in intracellular localization and possible function, we performed transfection studies with a series of green fluorescent protein (GFP)-tagged myosin-V fragments. The myosin-V domains were subcloned into pS65T-C1, a plasmid vector carrying the

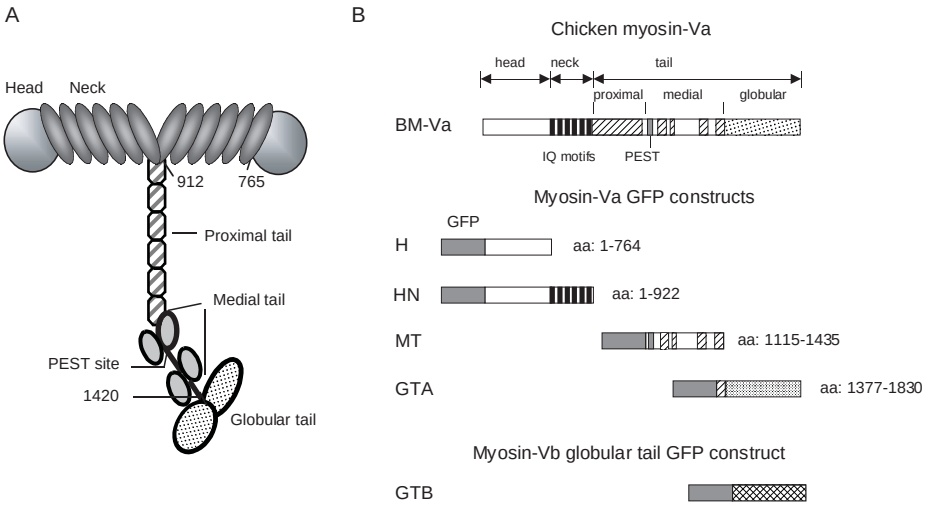

Fig. 1. (A) Schematic diagram of myosin-Va domain structure based on sequence analysis and electron micrographs. The numbers indicate amino acid positions at major junctions in the molecule. (B) Schematic diagram representing the primary structure of myosin-Va and the myosin-V GFP constructs. The amino acid positions are indicated. The start of the GTB construct corresponds to aa 1466 of BM-Va. The abbreviations on the left of each construct are used to refer to the GFP-myosin-V recombinant plasmids and the proteins they encode.

gene for green fluorescent protein upstream of the multicloning site, so as to encode fusion proteins of the inserted fragment with GFP at the NH_2 terminus. The clones were verified by restriction enzyme analysis and sequencing at the sites of ligation. Four constructs encoding domains of the chicken myosin-Va heavy chain were generated (Fig. 1B): (1) GFP-head (H), containing the sites for binding actin and ATP, (2) GFP-head-neck (HN), containing the head as well as the six IQ motifs responsible for light chain binding, (3) GFP-medial tail (MT), which includes the calpain cleavage-sensitive PEST site, globular subdomains, and a predicted coiled-coil α-helix, and (4) GFP-globular tail (GTA) coding for the distal globular domain. A construct encoding the globular tail of murine myosin-Vb (GTB) was also generated (Fig. 1B). The GFP-myosin-V constructs were transfected into cells of the murine melanoma lines B16 F-10 (wild type for myosin-Va) and S91-6 (derived from *dilute* mice). Except where specifically described, fluorescence staining results obtained with mutant cells were similar to those obtained in wild-type melanocytes

Previous studies have shown that treatment of melanocytes with α-melanocyte stimulating hormone results in a ~2.5 (Wu et al., 1997) to ~4-fold (Nascimento et al., 1997) increase in the expression of myosin-V. Consequently, we performed our transfection experiments on unstimulated cells to minimize the possibility that endogenous myosin-V might occupy all available ligand binding sites and thereby inhibit targeting of GFP-labeled fusion proteins.

GFP-myosin-Va head codistributes with actin filaments

Melanocytes transfected with the GFP-myosin-Va head (H) construct exhibit filamentous staining strikingly reminiscent of stress fibers (Fig. 2A). Localization of the H domain extended throughout the cytoplasm into cellular processes (Fig. 2A) but was excluded from the nucleus as confirmed by confocal microscopy. When transfected cells were fixed and stained with rhodamine phalloidin, H expression was found to colocalize with filamentous actin (Fig. 2B-D). This pattern of fluorescence was strikingly different than observed in transfected cells expressing the pS65T-C1 plasmid encoding GFP without a myosin insert. In these cells GFP fluorescence was diffusely distributed in a uniform manner throughout the cytoplasm and nucleoplasm (data not shown). When examined over time, we found that GFP (data not shown), and GFP-myosin-V-expressing cells (Figs 2-5) exhibited extended dendrites, motile melanosomes and dynamic filopodia, suggesting that the constructs used in this study were nontoxic

GFP-myosin-Va head-neck colocalizes with actin and is concentrated in cell extensions

GFP-myosin-Va head-neck (HN) expression exhibited a filamentous distribution in the cytoplasm similar to that observed for the H domain (Fig. 2E). However, unlike for the H domain, HN localization was often concentrated in the filopodia. Interestingly, the localization of HN in filopodia appeared to be cell-type-dependent. In B16 cells, intense fluorescence was observed as distinct dots at the distal ends of filopodia (Fig. 2E, insert) whereas in mutant S91-6 cells, the entire length of filopodia exhibited elevated HN staining (Fig. 2F, insert). Filopodia also had a wavy appearance compared to cells not expressing HN (Fig. 2F).

GFP-myosin-Va medial tail localization is diffuse and cytoplasmic

The staining pattern of GFP-myosin-Va medial tail (MT) was fairly homogenous throughout the cytoplasm but difficult to detect in filopodia (Fig. 3A). No fluorescent fibers or filopodial puncta were observed. Staining was excluded from the nucleus.

Expression of the myosin-V globular tail domains

We constructed a GFP-myosin-Vb globular tail plasmid (GTB) and a GFP-myosin-Va globular tail (GTA) plasmid and examined their expression in melanocytes. Compared to GTA, the peptide sequence of GTB is less similar to the corresponding region encoded by the *MYO5a* gene at the *dilute* locus in mice. Staining in GTB transfected cells was characterized by broadly distributed cytoplasmic puncta over an elevated background of diffuse fluorescence (Fig. 3B). In stark contrast, the earliest detectable fluorescent signal in cells expressing GTA was a distinct perinuclear dot suggestive of the centrosome (Fig. 4A). Phase contrast images of cells expressing GTA confirmed that localization was in fact perinuclear (Fig. 4B). Unlike for cells expressing high levels of GTB, fluorescence in cells expressing elevated levels of

GTA was observed as discrete puncta mostly in the vicinity of the nucleus (Fig. 5C) or as elongate shapes in the cell cytoplasm (Fig. 4E).

To confirm that GTA was localized at the centrosome, transfected cells were fixed and stained with anti-tubulin antibodies (Fig. 5). Immunofluorescence imaging of cells stained with anti β-tubulin (Fig. 5B) showed that the main locus of GTA fluorescence correlated with the microtubule organizing center (Fig. 5C). Staining for γ-tubulin (Fig. 5E), a protein known to be concentrated at the centrosome (Stearns et al., 1991), produced a localized and intense perinuclear patch that colocalized with the major locus of expressed GTA (Fig. 5F). Digital image analysis (MetaMorph, Universal Imaging Inc.) revealed that >98% of the GTA fluorescence in Fig. 5A was confined to a single perinuclear dot that was ~17-fold more fluorescent than the remaining cytoplasm.

To examine the effect of GTA expression on the localization of endogenous native myosin-V, cells transiently expressing GTA were fixed and immunostained with an antibody against the head domain of chicken brain myosin-Va that does not recognize GFP-GTA (Fig. 5G-I). In transfected cells, GTA expression was consistently observed to be most intense at the centrosome (Fig. 5G). Immunostaining with the anti-head domain antibody revealed that endogenous myosin-V was present throughout the cytoplasm in both transfected and untransfected cells. More importantly, in untransfected cells, endogenous myosin-V was highly concentrated as a distinct perinuclear dot consistent with centrosome staining (Fig. 5H).

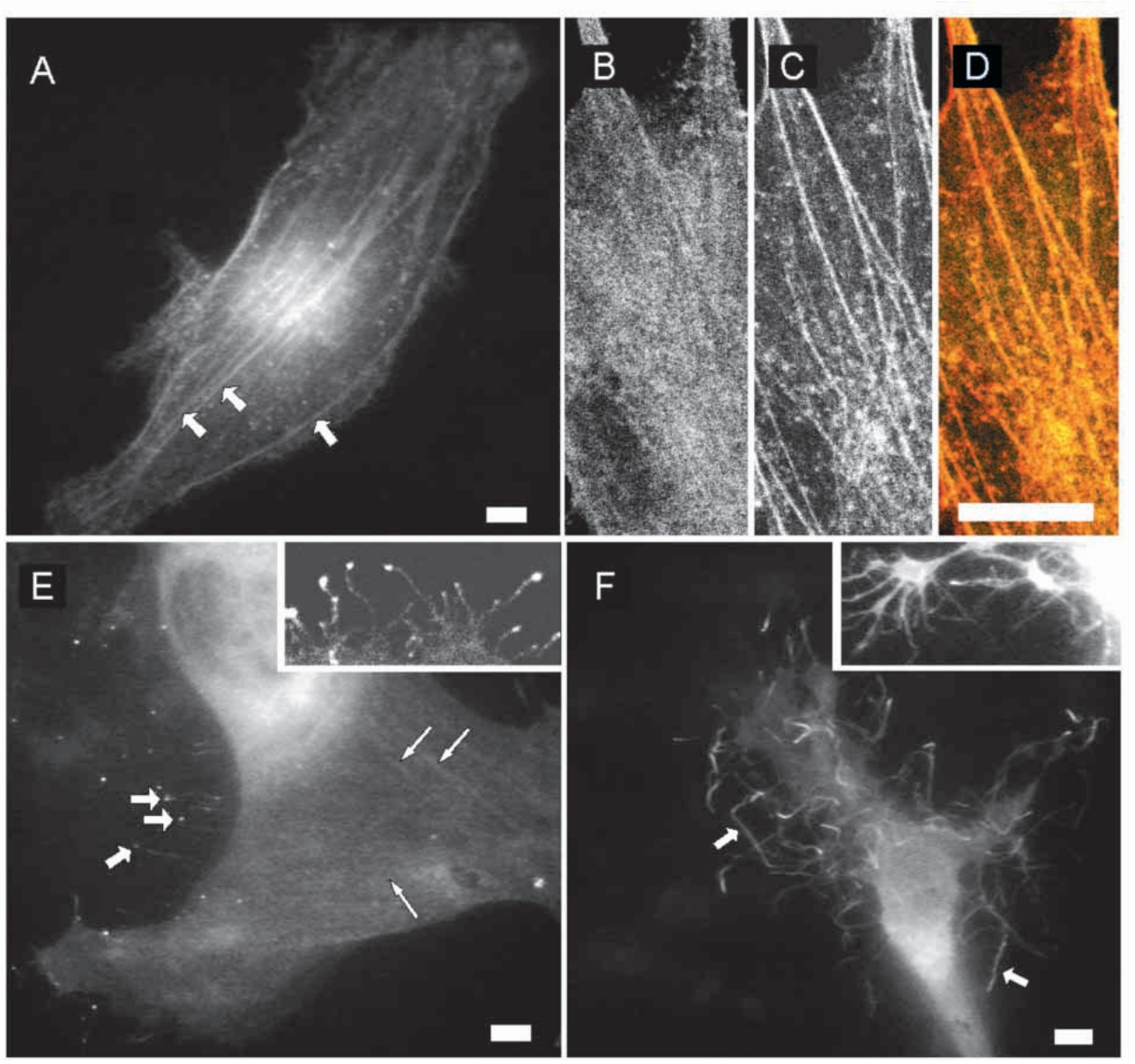

Fig. 2. Melanocytes expressing GFP-myosin head (H) and GFP-myosin head-neck (HN). (A) Expression of H in B16 cells. Note the filamentous staining reminiscent of F-actin (arrows). (B-D) Confocal images of a melanocyte showing fluorescence of H (B), staining of F-actin with rhodamine phalloidin (C), and the overlay showing colocalization of H with F-actin (D). (E) Expression of HN in B16 melanocytes. In wild-type cells HN is found on filaments (arrows) and concentrated at the tips of filopodia (large arrows, inset). (F) Expression of HN in S91 mutant cells is uniformly distributed throughout the elongate filopodia (arrows, inset). Bars, 10 μm.

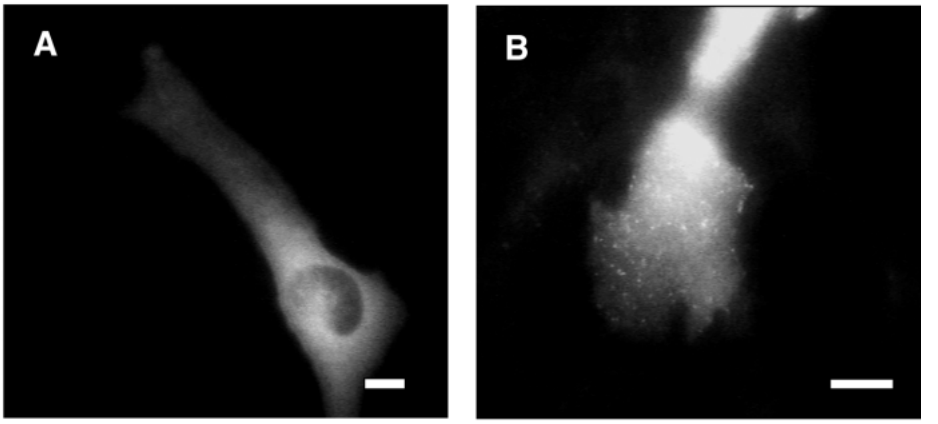

Fig. 3. (A) B16 melanocyte expressing the GFP-medial tail (MT) construct of myosin-Va. Expression of MT is diffuse throughout the cytoplasm and excluded from the nucleus. (B) Expression of the GFP-globular tail of myosin-Vb (GTB) in B16 melanocytes. GTB exhibits punctate cytoplasmic localization over a background of diffuse fluorescence. Bars, 10 μm.

In contrast, in GTA expressing cells the centrosomal localization of endogenous myosin-V was markedly reduced to levels found throughout the remainder of the cytoplasm (Fig. 5H,I). Hence, transient expression of the globular tail domain caused displacement of endogenous myosin-V from the centrosome.

Effects of nocodazole and cytochalasin D on centrosomal myosin-V

Given that the motor domain of myosin-V is not required for targeting to the centrosome (Fig. 5), we sought to determine whether actin filaments or microtubules were necessary for perinuclear localization of this motor molecule. To examine the role of microtubules, melanocytes were incubated with 20 μg/ml nocodazole for 1-24 hours, and then fixed and immunostained for myosin-V and β-tubulin. We found that, over time, this concentration of nocodazole resulted in a dramatic redistribution of myosin-Va, yet a sufficient number of microtubules were detectable that served as landmarks for identification of the centrosomal region. Confocal sections (0.5 μm per step in Z-axis) were then performed on treated cells stained for myosin-Va. When compared to melanocytes treated with DMSO alone (Fig. 6A), exposure to nocodazole resulted in loss of the large myosin-V dot that was formerly associated with the centrosome. Moreover, the intensity of myosin-V staining at the centrosome was markedly reduced to levels comparable to the rest of the cytoplasm (Fig. 6D).

To address the role of filamentous actin in centrosomal targeting of myosin-V, melanocytes were treated with either 0.2% DMSO (control conditions) or incubated with 10 μg/ml cytochalasin D (CD) for up to 24 hours. Cells were then fixed and stained for myosin-V, β-tubulin and F-actin. Actin filaments were clearly present in the perinuclear region of untreated cells, but they were not noticeably concentrated near the puncta of centrosomal myosin-V (Figs 7C, 8A). Incubation with CD resulted in the rapid depolymerization of most of the F-actin, and the perinuclear region was completely devoid of actin filaments by 30 minutes of treatment. In contrast to nocodazole-treated cells, at no time point did we observe diminution of centrosomal myosin-V staining in cells exposed to CD (Fig. 7). Remarkably, in cells incubated for 6 hours with CD, staining of centrosomal myosin-V was almost always more intense than observed in untreated cells (compare Fig. 8A and B). In melanocytes treated with CD for 3-6 hours, an intense dot of centrosomal myosin-V staining was observed in >97% (102 of 105) of interphase cells scored. The radial area surrounding the centrosome was typically depleted of myosin-V and F-actin following treatment with CD (Figs 7D,F, 8B). Interestingly, myosin-Va staining was typically observed to colocalize with the CD-resistant actin filaments that formed an intense ring at the cell periphery (Fig. 7C).

It has been reported previously that microtubules become resistant to the effects of nocodazole over time (Lillie and Brown, 1998). To examine further the role of microtubules in centrosomal localization of myosin-V, we examined melanocytes treated for prolonged time periods with nocodazole (e.g. >5 hours) or exposed to cold followed by treatment with nocodazole (90 minutes). In those cells in which microtubules were present (presumably due to repolymerization), several puncta of myosin-V were detected at the centrosome (Fig. 8C) and associated with microtubules (Fig. 8D). In all interphase cells examined, the centrosomal myosin-V staining in nocodazole-treated cells was markedly less than observed for CD-treated cells (compare Fig. 8C to B).

DISCUSSION

There is compelling evidence based on myosin-V mutations in mouse (*dilute*) and yeast (*Myo2-66*), together with immunolocalization studies of chick brain myosin-V (BM-Va), to suggest that this class of myosins has a role in organelle/vesicle transport and growth of cellular extensions (Johnston et al., 1991; Mercer et al., 1991; Espreafico et al., 1992; Govindan et al., 1995; Wang et al., 1996; Evans et al., 1998). A popular speculation which often appears in models of myosin-V function shows the catalytic heads walking down actin tracks toward the barbed end of filaments while pulling cargo and/or membranes associated with the COOH-terminal tail. However, recent evidence by Wu et al. (1998b) suggests that in the case of melanosomes, myosin-Va functions as a dynamic 'capture' molecule, which results in distal accumulation of these large pigmented organelles. Here we show that expression of motor-containing domains of the myosin-Va heavy chain in live mouse melanocytes localize to sites that are consistent with a role for this mechanoenzyme in actin-based transport and organelle localization. We also provide evidence that differences in class-Va and Vb myosin tail domains result in differential subcellular localization. Myosin-Va localization to the centrosome is specific when

assessed using a competitive binding assay, and centrosome association occurs independent of the actin-binding motor domain. Of significance, we demonstrate that maintenance of myosin-Va at the centrosome does not require intact actin filaments, but does depend on microtubules.

Myosin-V head and head-neck domains bind endogenous actin filaments

Our experiments designed to look at the subcellular distribution of myosin-V heavy chain domains demonstrate that the head (H) and the head-neck (HN) domains bind endogenous cytoplasmic actin. The HN construct, which had the catalytic domain and the six IQ motifs known to bind light chains (Espreafico et al., 1992; Cheney et al., 1993) exhibited intense localization in the actin-rich filopodia in a cell-dependent manner. HN was concentrated along the entire length of filopodia in S-91 cells derived from *dilute* mutants. In contrast, HN was concentrated only at the tips of filopodia in B-16 cells wild type for the *dilute* gene product.

The cellular details involved in concentrating HN to filopodia (compared to the H peptide) are unknown. Perhaps the HN peptide is preferentially localized to filopodia due to interactions of the neck domain (and/or associated light chains) with vesicles or particles in route to the cell periphery. Interestingly, myosin-V in the periphery of nerve growth cones is associated with a distinct population of small organelles (50-100 nm) and with actin filaments and the plasma membrane (Evans et al., 1997).

Differences in the filopodial distribution of the HN peptide observed in wild-type and mutant melanocytes could be due to variations in the levels of cytoskeletal and/or filopodial proteins in these cells. Dissimilar expression patterns of actin, myosin, tubulin and vimentin have been reported when comparing the

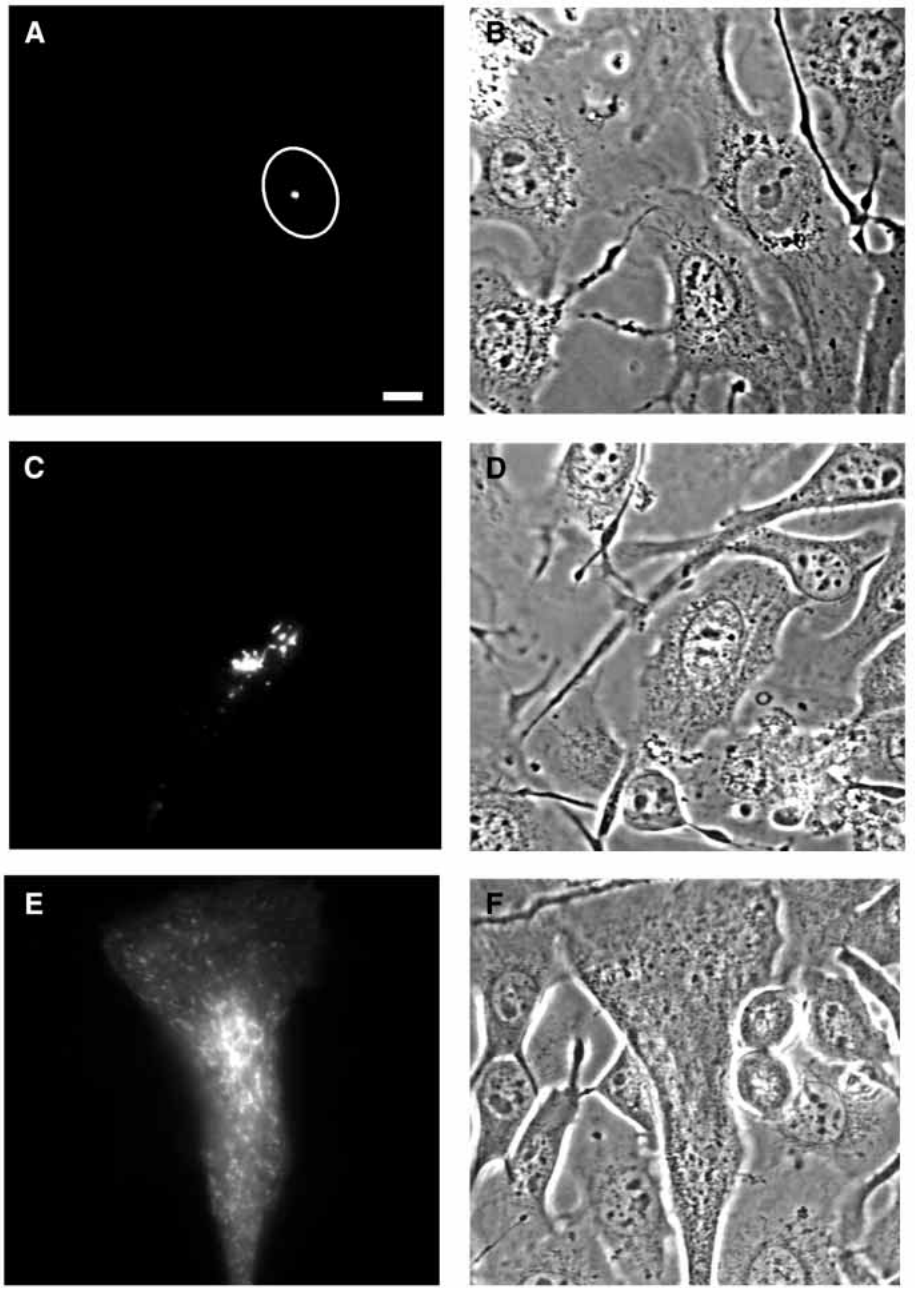

Fig. 4. Expression of the GFP myosin-Va globular tail domain in B16 cells is distributed primarily to one perinuclear spot. (A) GTA expression localizes to a distinct perinuclear point. The nucleus is outlined. (B) Phase contrast image of the same field. Note that in cells expressing GTA as an intense perinuclear dot, the globular tail peptide is not concentrated in or around melanosomes (melanosomes are small dark organelles visible throughout the perinuclear region of the transfected cell in 5B). (C) GTA expression occasionally localizes as additional puncta in the perinuclear region. (D) Phase contrast image of the same field. (E) GTA expression was also observed as elongate shapes, which did not correlate to any visible structures under phase contrast microscopy (F). Bar, 10 μm.

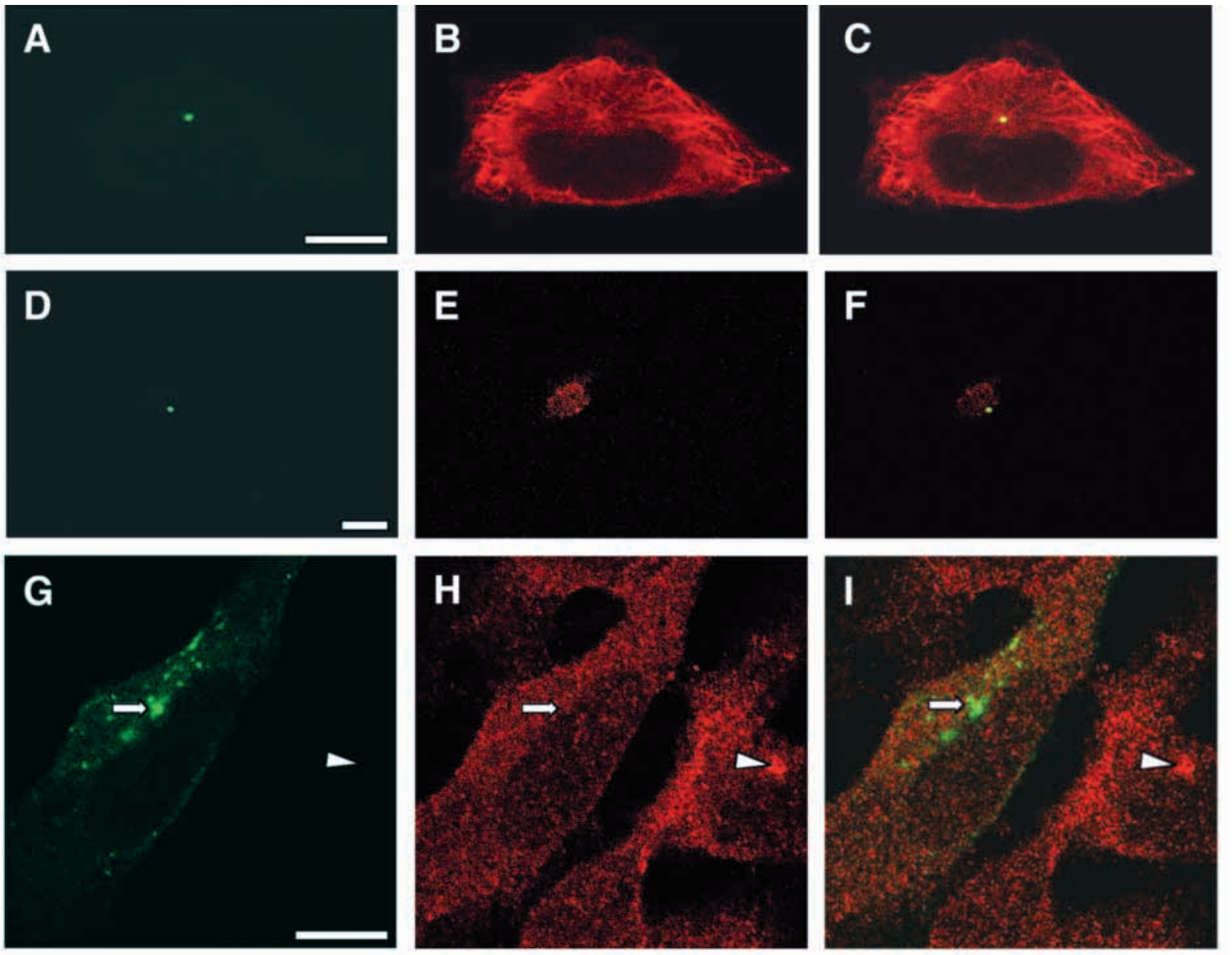

Fig. 5. The globular tail domain localizes myosin-Va to centrosomes. Each row corresponds to a distinct field. (A,D,G) Melanocytes transfected with GTA. Note that GTA expression is often localized to a distinct perinuclear dot. (B,E,H) Immunofluorescence staining with β-tubulin (B), γ-tubulin (E), and anti-head myosin-V antibodies (H). (C,F,I) Overlays of the GTA staining (green) and the antibody fluorescence (red) present in first two columns. (G-I) The arrow marks a GTA-labeled centrosome in a transfected melanocyte. The arrowhead marks the characteristic dot of endogenous myosin-V (stained with α-BM-V head-directed antibodies that do not recognize GTA) present at the centrosome of an untransfected melanocyte. Note that antibody staining of endogenous myosin-V is abolished at the centrosome in transfected cells expressing GTA (arrow in I). (A-C) S91-6 melanocytes and (D-I) B16 melanocytes. Bars, 10 μm.

central area to the periphery of the same tumor (Puches et al., 1991). Since B16 cells and S91-6 cells are derived from separate tumors, they are likely to have different protein expression profiles. Furthermore, actin expression is markedly decreased in melanoma cells (compared to normal melanocytes) when assayed for mRNA levels (Gomez et al., 1995) and by immunohistochemical analysis of benign and malignant melanocytic skin tumors (Puches et al., 1991). Immunoblots of Cloudman S91-6 cell homogenates probed with antibodies to chick brain myosin-Va demonstrate barely detectable amounts of the myosin-V immunogen when using total protein loads that provide strong detection of myosin-V from B16 cell homogenates (A. A. C. Nascimento, personal communication). Thus, if mutant S91-6 cells have subsaturating amounts of actin-binding proteins, the HN peptides might be free to interact with myosin-binding domains located along the entire length of filopodia actin filaments. In contrast, in wild-type cells, the normal complement of cellular proteins would act to regulate the localization of myosin-Va to more specific intracellular regions, such as to the tips of filopodia (see Fig. 3E). Interestingly, immunofluorescence localization studies show myosin-V is also localized to the tips (as well as in the terminal web) of brush border microvilli in avian enterocytes (Heintzelman et al., 1994). These data support the idea that localization of myosin-Va to the tips of actin-rich cellular processes occurs independently of the tail domain.

A third possibility is that the GFP-motor peptides exhibit distinct mechanochemical properties (e.g. regulated versus unregulated motor activity). If the motor-containing myosin peptides were able to translocate along actin cables, their distribution could be differentially affected by tropomyosins. Tropomyosin-coated actin filaments have recently been demonstrated to direct Myo2p (myosin-V) enrichment to sites of bud emergence in yeast (Pruyne et al., 1998).

The globular tail of class-Va myosins colocalizes with centrosomes

Transient expression of the GFP-myosin-Va globular tail

(GTA) was consistently observed to colocalize with the centrosome as confirmed by immunofluorescence of transfected cells using antibodies to β- and γ-tubulin (Fig. 5). This finding is consistent with earlier immunofluorescence microscopy studies demonstrating that native myosin-Va encoded by the *MYO5a* gene is localized to a perinuclear dot (Nascimento et al., 1997), later confirmed to be the centrosome (Espreafico et al., 1998). We have extended these earlier studies by demonstrating that expressed GTA physically displaces native myosin-Va from centrosomes in living melanocytes. Hence GTA localization to centrosomes is likely a consequence of binding to a specific target, rather than due to adventitious association with an abundant centrosomal component(s). Sequence comparisons of the residues in the globular tail of chick brain myosin-Va and the mouse *MYO5a* gene product indicate these domains are 96% identical (416 of 438 residues). Given this sequence similarity, and the displacement results observed in transfected cells, both mouse and chick class-Va myosins appear to recognize a common ligand at the centrosome.

Expression of GTA was also evident in some cells as puncta in the vicinity of the nucleus (Fig. 4C,E) and as straight lines strikingly similar to microtubules (data not shown). This localization pattern may be due to association of GTA with perinuclear organelles aligned with microtubules, including mitochondria, endoplasmic reticulum and Golgi networks, organelles previously demonstrated by immunogold electron microscopy to colocalize with myosin-V (Nascimento et al., 1997). Unlike in previous localization studies of myosin-V (Nascimento et al., 1997; Wu et al., 1997, 1998) we did not observe a striking overlap between any of the GFP-myosin-Va domains with melanosomes (data not shown). Digital overlays of phase contrast (to visualize melanosomes) and fluorescence images of GTA- and GTB-expressing cells showed limited colocalization between these fusion proteins and melanosomes (data not shown), but the overlap was markedly less than in a previous study based on indirect immunofluorescence localization of endogenous myosin-Va (Nascimento et al., 1997).

We have thus far been unable to compare the localization pattern of GFP-full-length myosin-Va with the distribution of the heavy chain domains. In a preliminary study using indirect immunofluorescence detection of full-length myosin-Va (without the GFP tag) expressed in mutant melanocytes lacking endogenous myosin-Va; Bizario et al. (1998) reported partial colocalization of myosin-Va heavy chain with melanosomes. Interestingly, intense staining was observed throughout the perinuclear region, suggesting that full-length myosin-Va also targets to the centrosome. However a distinct centrosomal dot (see Fig. 4A) was not detected, presumably because the

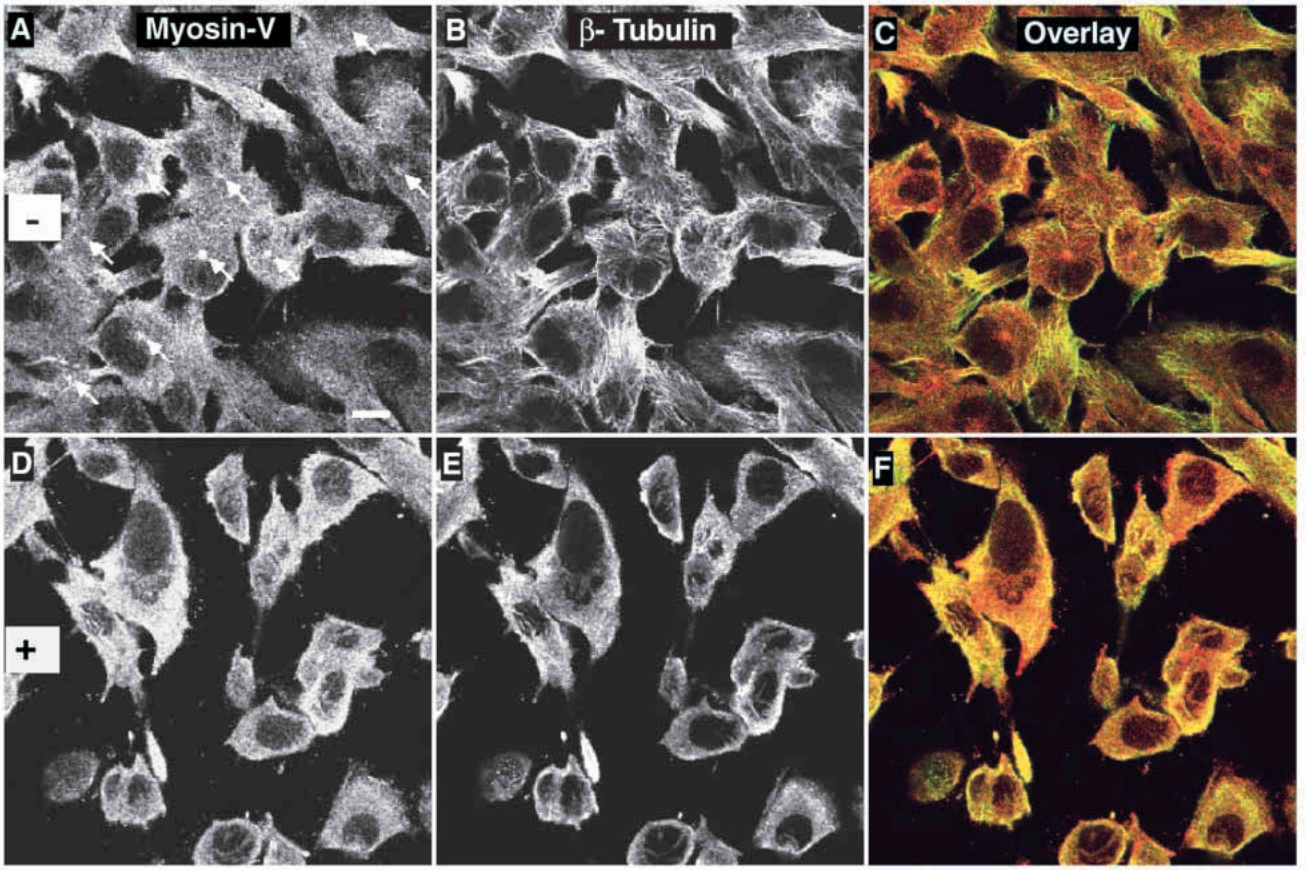

Fig. 6. Myosin-V localization at the centrosome requires intact microtubules. (A-C) B-16 melanocytes in the absence (–) of nocodazole. (D-F) Melanocytes incubated with (+) 20 μg/ml nocodazole for 3.5 hours. Immunostaining for myosin-V (A,D), β-tubulin (B,E) and their respective overlays (C,F). Note that in (A), the myosin-V staining in untreated cells is clearly localized to distinct perinuclear dots (arrows) that correspond to the centrosome (B,C). In contrast, cells treated with nocodazole do not have localized foci of myosin-V in the perinuclear region (D). Very few microtubules are visible following exposure to nocodazole (E). Confocal images. Bar, 10 μm.

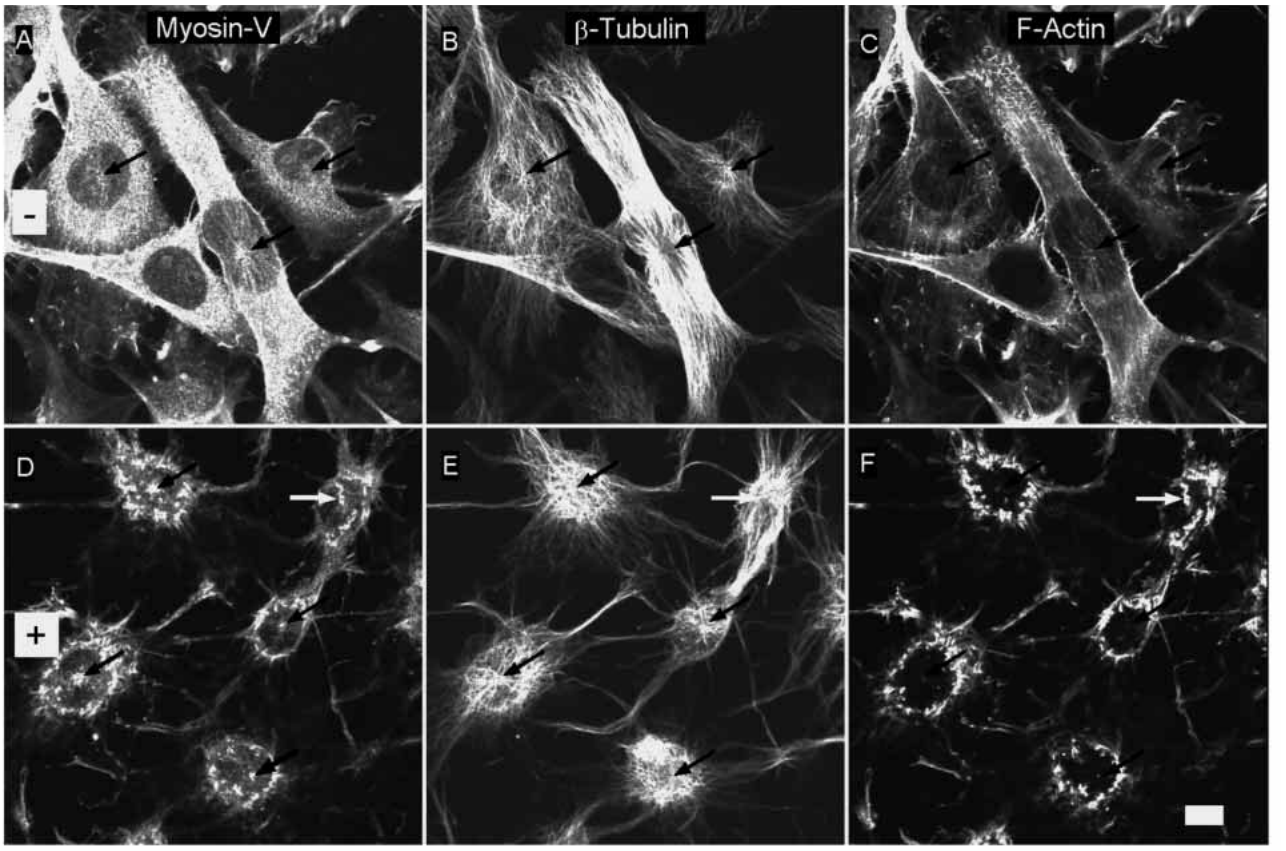

Fig. 7. Effects of cytochalasin D on myosin-V localization. (A-C) Untreated B-16 melanocytes. (D-F) Melanocytes treated with 10 ug/ml CD for 3 hours. Staining of cells for myosin-V (A,D) β-tubulin (B,E) and F-actin (C,F). Arrows indicate the centrosome-containing region identified at the center of the radiating microtubules. Myosin-V is localized to this region in untreated (A) and CD-treated cells (D). Treatment with CD results in two distinct alterations: myosin-V at the centrosome appears to be organized as a denser locus (D), and the area surrounding the centrosome is devoid of myosin-V and filamentous actin (F). The white arrows (D-F) indicate one exception where filamentous actin is colocalized to the centrosome following treatment with CD. Confocal images. Bar, 10 μm.

fluorescence at the centrosome could not be distinguished from the high levels of expressed full-length myosin-Va heavy chain found throughout the perinuclear region. Expression of full-length myosin-Va heavy chain was observed within filopodia in B16 melanocytes (Bizario et al., 1998). However this peptide was not observed to be concentrated in the tips of filopodia, as was the case for the GFP-HN domain of myosin-Va (Fig. 2E). Full length myosin-Va heavy chain also exhibited diffuse staining throughout the cytoplasm (Bizario et al., 1998), but did not display intense colocalization with F-actin, as was observed for the GFP-H and GFP-HN peptides (Fig. 2). These localization differences observed between full-length myosin-Va compared to the GTA, H and HN domains suggest that proper targeting of myosin-Va requires a full-length molecule. Native myosin-Va localization appears to be dependent on interactions of the motor domain with F-actin as well as association of the GTA domain with its receptor (e.g. docking molecule).

Other differences in the localization of myosin-Va are likely to result from changes in intracellular expression levels (compare Fig. 4A and C), or due to cell cycle-dependent alterations in the cytoskeleton. Differences in the observed immunofluorescence localization of myosin-V are not uncommon. Wu et al. (1997) found that myosin-V was localized very specifically with melanosomes in stimulated melanocytes, but unlike for Nascimento et al. (1997), staining was undetected in centrosomes, Golgi and ER.

In contrast to GTA, neither the medial tail (MT) nor the globular tail domain from a murine class-Vb myosin (GTB) were localized to a distinct perinuclear dot (Fig. 3). Expression of MT and GTB was diffuse throughout the cytoplasm. Puncta of GTB were also present throughout the cytoplasm, but they were not concentrated near the nucleus. Thus, the primary sites of GTB expression appear to be distinct from those observed for GTA.

We did identify filamentous actin near the centrosome in melanocytes stained with rhodamine-phalloidin (Figs 7C, 8A). This is consistent with previous findings demonstrating that actin is abundant in centrosomes isolated from *Spisula* (Vogel et al., 1997) and human lymphoid cells (Bornens et al., 1987), and is necessary for determining centrosome position (Euteneuer and Schliwa, 1985). However, our data do not support a role for actomyosin-based motility in localization of myosin-Va to the centrosome. For example, the demonstration that GTA (but not H or HN) localizes to the centrosome in the absence of the NH_2-terminal motor domain makes it highly unlikely that the mechanochemical activity of myosin-Va functions in perinuclear localization. The possibility that

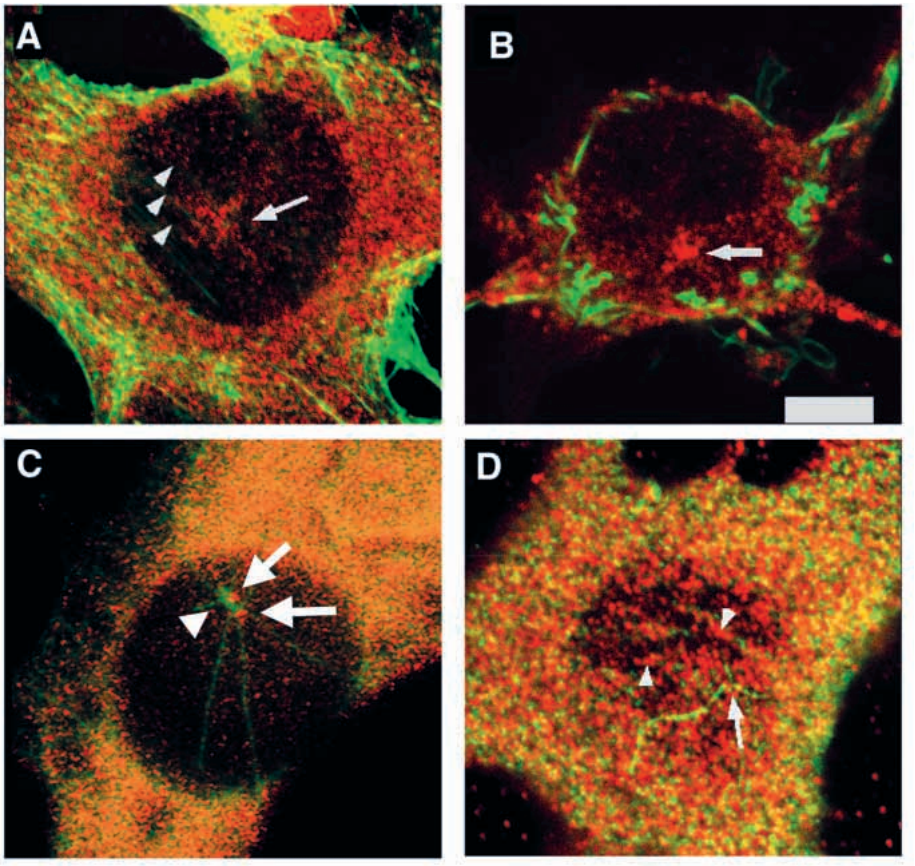

Fig. 8. Centrosomal myosin-V localization is differentially affected by prolonged treatment of melanocytes with CD or nocodazole. (A) Representative melanocyte stained for F-actin (green) and myosin-V (red) in the absence of cytoskeletal drugs. Actin filaments (arrowheads) passing through the perinuclear region are not concentrated where centrosomal myosin-V is localized (arrow). (B) Representative melanocyte treated with 10 ug/ml cytochalasin D for 6 hours and stained for F-actin (green) and myosin-V (red). Note that even after prolonged exposure to CD, myosin-V remains highly concentrated as a perinuclear dot (arrow). Actin filaments are completely absent from this region. (C) Melanocyte incubated at 4°C for 30 minutes followed by treatment with nocodazole at 37°C for 90 minutes. Note that most of the microtubules are absent, and only two distinct myosin-V puncta (arrows) are colocalized with the centrosome (arrowhead). (D) Representative melanocyte stained for β-tubulin (green) and myosin-V (red) following treatment with 20 μg/ml nocodazole for 6 hours. Several microtubules are associated with puncta of myosin-V (arrowheads). Note the microtubules radiating from a perinuclear point (arrow), suggesting proximity to the centrosome. Confocal images. Bar, 5 μm.

actomyosin motility may play a role in delivery to the centrosome cannot be completely excluded since myosin-Va may be a component of a complex that is transported by one of the many other myosins present in vertebrate cells. However, this possibility is seriously weakened by our finding that during prolonged maintenance of cells in conditions that inhibit actin filament formation (10 μg/ml CD), the centrosomal myosin-Va typically becomes more highly concentrated as a well-defined dot compared to untreated cells (compare Fig. 7A and D). For this same reason, actin filaments do not appear to be required for the continued association of myosin-Va with the centrosome over time. Interestingly, treatment with CD causes a redistribution of cytoplasmic myosin-V to the cell periphery where it is highly colocalized with the drug-resistant F-actin (Figs 7D,F, 8B). This suggests that actin filaments are at least partially responsible for proper localization of cytoplasmic myosin-V.

The position of the centrosome relative to microtubules raises the exciting possibility that these cytoskeletal elements facilitate the perinuclear localization of myosin-Va. Myosin-V has been demonstrated to colocalize with microtubules in the central domain of rat nerve growth cones (Evans et al., 1997) and with microtubules in dividing cells (Wu et al., 1998a). No evidence exists for direct binding of myosin-Va to microtubules in vivo, and purified chick brain myosin-Va does not bind purified microtubules in a cosedimentation assay (Cheney et al., 1993). Hence, any possible association of myosin-Va with microtubules is likely to be indirect.

There is sufficient evidence to speculate that myosin-Va may be transported as part of a small vesicle or particle that is localized to the centrosome by microtubule motor proteins. For example, Espindola et al. (1996) found that BM-Va contains an 8 kDa dynein light chain that binds to sites within the COOH-terminal tail domain. Dynein is a minus-end directed microtubule motor that tethers centrosomes to spindle poles and is required for microtubule organization into poles (Heald et al., 1997). Links between myosin and microtubule motors were strengthened further by Rogers and Gelfand (1998), who found that melanophores isolated from *Xenopus* have myosin-V as well as bound plus-end (kinesin-II) and minus-end directed microtubule motor proteins. Kinesins constitute a superfamily of microtubule motors important in organelle transport and centrosome function (see reviews by Kashina et al., 1997; Hirokawa, 1998). Both minus-end kinesin-like proteins (Kuriyama et al., 1995) and plus end-directed kinesins (Boleti et al., 1996) have been localized on centrosomes. The recent discovery by Huang et al. (1999) demonstrating that myosin-Va binds directly to conventional kinesin suggests a mechanism by which this myosin can be transported away from the centrosome toward the fast growing ends of microtubules. No interaction of myosin-Va with kinesin-related microtubule motors was observed in that report (Huang et al., 1999). Hence additional studies are needed to identify targets of myosin-V that are responsible for minus-end directed microtubule motility and association with the centrosome.

Taken together these data provide evidence that class-V myosins perform discrete functions and that their intracellular localization is modulated by the head (motor) and globular tail domains as well as by actin filaments and microtubules. It is plausible to speculate that myosin-V localized at the

centrosome may function to organize actin filaments in a cell-cycle dependent manner or as a dynamic bridge indirectly linking microtubules to actin or membranes. Considering that melanocytes from *dilute lethal* mice divide at half the rate of wild-type melanocytes (Provance et al., 1996), cell-cycle analysis of myosin-V and its centrosomal ligands will be an important future area of investigation.

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