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Myosin V associates with melanosomes in mouse melanocytes: evidence that myosin V is an organelle motor

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SUMMARY

Mice with mutations at the *dilute* locus exhibit a 'washed out' or 'diluted' coat color. The pigments that are responsible for the coloration of mammalian hair are produced by melanocytes within a specialized organelle, the melanosome. Each melanocyte is responsible for delivering melanosomes via its extensive dendritic arbor to numerous keratinocytes, which go on to form the pigmented hair shaft. In this study, we show by light immunofluorescence microscopy and immunoelectron microscopy that the myosin V isoform encoded by the *dilute* locus associates with melanosomes. This association, which was seen in all mouse melanocyte cell lines examined and with two independent myosin V antibodies, was evident not only within completely melanized cells, but also within cells undergoing the process of melanosome biogenesis, where coordinate changes in the distributions of a melanosome marker and myosin V were seen. To determine where myosin V, a known actin-based motor, might play a role in melanosome transport, we also examined the cellular distribution of F-actin. The only region where myosin V and F-actin were both concentrated was in dendrites and dendritic tips, which represent the sole destination for melanosomes and where they accumulate in cultured melanocytes. These results support the idea that myosin V serves as the motor for the outward movement of melanosomes within dendritic extensions, and, together with the available information regarding the phenotype of mutant melanocytes in vitro, argue that coat color dilution is caused by the absense of this myosin V-dependent melanosome transport system.

Key words: Myosin V, Melanosome, Organelle Motility

INTRODUCTION

Mice with mutations at the *dilute* locus, which encodes the heavy chain of a type V unconventional myosin (Mercer et al., 1991), exhibit a defect in hair coloration (Jackson, 1994; Silvers, 1979). This coat color defect is seen in all of the over 200 recessive mutant *d* alleles identified to date. These mutations include a single allele associated with the integration of an ecotropic murine leukemia virus into the *dilute* locus (Jenkins et al., 1981), which is referred to as *dilute viral* or *dv*, and a large number of spontaneous and induced alleles, which are referred to collectively as *dilute lethal* or *dl* because they also exhibit apparent neurological defects that result in the death of juvenile animals (Strobel et al., 1990). The coat color defect caused by these alleles is very evident on a nonagouti background, where the coat appears gray instead of black, but *d* alleles also cause the 'dilution' of brown and yellow coats.

The pigments (melanins) that are responsible for the coloration of mammalian hair (and skin) are produced within melanocytes, which are dendritic cells of ectodermal origin that reside near the base of the hair bulb and within the bottommost layer of the epidermis (for review, see Boissy, 1988; Hearing and King, 1993; Jimbow et al., 1993). These cells are responsible for delivering pigment, via their extensive dendritic arbor, to numerous keratinocytes, the principal cell type of hair and skin, as these keratinocytes migrate into the hair shaft proper and to the skin surface. The synthesis of melanins occurs entirely within a specialized organelle of the melanocyte, the melanosome. Melanosomes first appear in the central cytoplasm as pigmentless vesicles containing prominent internal striations. These premelansomes, as they are often called, gain the capacity to synthesize melanin only after the incorporation into their limiting membrane of several melanogenic enzymes, one of which is tyrosinase-related-protein-1 (TRP1). At this point, melanin polymer begins to accumulate, leading to melanosomes in which internal structure is at first partially obscured (lightly melanized), and then fully obscured (fully melanized). These latter melanosomes, which appear completely black in both light and electron micrographs, are the end product that melanocytes provide keratinocytes. This process of melanosome maturation is coupled with the movement of the organelle from the cell body to the tips of the cell's dendrites, which appear to be the sole sites of melanosome transfer to keratinocytes.

Previous studies have shown that *dilute* mice contain normal numbers of follicular and epidermal melanocytes, that these melanocytes synthesize normal amounts of melanin pigment, and that melanosome biogenesis is normal at the ultrastructural

level in *dilute* melanocytes (for review, see Silvers, 1979; Jackson, 1994). These facts, and the fact that essentially all of the visible coloration of mammalian hair and skin requires the transfer of melanosomes to keratinocytes, together suggest that coat color dilution is caused by a myosin V-related defect somewhere between the biogenesis of this organelle and its transfer to keratinocytes. One candidate defect, which has gained widespread acceptance in the literature as the underlying cause of coat color dilution, is that *dilute* melanocytes are adendritic (reviewed by Silvers, 1979). The aberrant distribution of pigment evident in unstained preparations of mutant skin, hair follicles and Harderian gland (clumped rather than dispersed), which has formed the basis for this conclusion, can also be explained, however, by a defect in the outward transport of melanosomes within melanocytes of normal shape. Either type of defect would lead to abnormalities in the transfer of melanosomes to keratinocytes, resulting in coat color dilution. It is not clear from images of unstained tissues, therefore, whether myosin V functions in melanocytes in the formation and/or maintenance of polarized cell shape, or in melanosome transport.

In an effort to further define the function of myosin V in melanocytes, we sought in this study to determine by immunolocalization whether myosin V associates with melanosomes. To accomplish this, we generated two polyclonal antibodies against different portions of the myosin V heavy chain encoded by *dilute*. These isoform-specific myosin V antibodies were used in conjunction with phase microscopy, which revealed the positions of heavily-melanized melanosomes, and antibodies against TRP1, which revealed not only end-stage melanosomes, but lightly-melanized melanosomes and compartments involved in the delivery of TRP1 to melanosomes (both of which are invisible in phase images). These antibodies, together with antibodies to various membrane compartments and a fluorescent probe for actin filaments, were used to stain a variety of mouse melanocyte cell lines, including a transformed mouse melanoma cell line (B16-F10) and two nontransformed but immortal mouse melanocyte cell lines (B10BR and melan-a). Our finding that myosin V does associate with melanosomes supports the idea that the coat color defect is caused by the absence within *dilute* melanocytes of a myosin V-dependent melanosome transport system. These results link a well-characterized unconventional myosin (Brokerhoff et al., 1994; Cheney et al., 1993; Espreafico et al., 1992; Govidan et al., 1995; Johnston et al., 1991; Larson et al., 1990; Lillie and Brown, 1994; Mercer et al., 1991; Mooseker and Cheney, 1996; Wolenski et al., 1995) with a known organelle. Furthermore, this connection is made within a cell type where it now appears from analyses of the phenotype of *dilute* melanocytes in vitro that the absence of this myosin results in a significant disruption in the transport of the organelle in vivo (Koyama and Takeuchi, 1981; Provance et al., 1996; Wei et al., 1997), thereby lending additional support to the idea that myosin V serves as an organelle motor in these cells.

MATERIALS AND METHODS

Melanocyte cell lines

The mouse melanoma cell lines B16-F10 (*D/D,B/B*) and S91 (d^v/d^v,*b/b*) were obtained from Dr Vince Hearing (NCI, NIH) and the American Type Culture Collection (CCL 53.1), respectively, and were grown in low-tyrosine Ham's F10 medium (American Bioorganics, #N-6100) supplemented with 10% horse serum (Gibco, #26050-013), 2% fetal calf serum (Gibco, #16140-063) and 1% (v/v) penn/strep (Gibco, #15070-014). The nontransformed but immortal mouse melanocyte cell line B10BR, which was isolated from the skin of a B10BR (*D/D, B/B*) mouse (Tamura et al., 1987), was kindly provided (in its 26th passage) by Dr Ruth Halaban (Yale University, New Haven, CT), and was cultured in the same medium as above, except with the addition of 85 nM tetradecanoyl phorbol acetate (TPA) (Sigma, #P8139) and 0.1 μM dbcAMP (Sigma, #D0627). The nontransformed but immortal mouse melanocyte cell lines melan-a (*D/D, B/B, C/C*), melan-b (*D/D, b/b, C/C*), and melan-c (*D/D, B/B, c/c*) (Bennet et al., 1987, 1989) were obtained from Dr Dorothy Bennett (St George's Hospital Medical School, London) and were cultured in minimum essential medium (Gibco, #41600-016) supplemented with 5% fetal calf serum, 1% (w/v) sodium pyruvate, 700 μM sodium bicarbonate, 0.7 μM 2-mercaptoethanol, 1% (v/v) penn/strep, and 0.2 μM TPA. To induce pigmentation in B16-F10 cells, alpha-melanocyte-stimulating hormone (Calbiochem, # 05-23-075) was added at a final concentration of 0.2 μM.

Primary melanocyte culture

Short-term cultures of primary melanocytes were prepared from the epidermis of individual newborn mice by the method of Tamura et al. (1987). Control melanocytes were prepared from the skins of C57BL/6J (*D/D*) pups, while *dilute* null melanocytes were prepared from the skin of C57BL/6J pups that were homozygous for the *dilute lethal* allele d^{l20J} (Strobel et al., 1990). These latter pups were obtained by crossing heterozygous parents of the genotype $d^{l20J}/d^v se$ (a generous gift from Dr Neal Copeland and Dr Nancy Jenkins, NCI, NIH), and were distinguished from their $d^{l20J}/d^v se$ and $d^v se/d^v se$ littermates based on western blot analyses performed using the myosin V antibody PDIL-1 on whole brain extracts prepared from pups at the time of sacrifice for primary melanocyte cultivation. Only the brains of homozygous d^{l20J} pups are devoid of detectable myosin V heavy chain protein (see Fig. 1).

Antibodies

To generate the *Escherichia coli* fusion proteins that were used to raise myosin V antibodies, a cDNA clone that spans the C-terminal ~75% of the *dilute* heavy chain was isolated from a mouse brain λgt10 cDNA library. Antibody DIL-1 was raised against a TRPE fusion protein containing myosin V heavy chain residues 1,259 to 1,853 (numbering from Mercer et al. (1991), except that residues 1,387-1,411 (exon F in Seperack et al., 1995), which are not normally present in the major spliced isoform in brain, were not in our cDNA). Antibody DIL-2 was raised against a GST fusion protein containing myosin V heavy chain residues 910-1,106, which correspond to the first segment of α helical coiled-coil in the central rod domain. The generation and purification of these fusion proteins, the immunizations of rabbits, and the purification of crude DIL-1 serum by repeated adsorption against nitrocellulose filters to which whole cell extracts from S91 melanocytes were bound (minus the region from ~185 kDa to ~195 kDa), were performed as described previously (Jung et al., 1993, 1996). The minimum concentrations of PDIL-1 and DIL-2 that were necessary to give bright staining of B16-F10 cells were determined by titration (~1:10 for PDIL-1, which corresponds to an ~1:400 dilution of DIL-1 serum when one includes the ~40-fold dilution involved in purifying DIL-1; ~1:300 for DIL-2). Rabbit polyclonal antibodies to mannosidase II and TRP1 (PEP1) (Jimenez et al., 1989) were gifts from Dr Kelly Moreman (University of Georgia, Athens, GA) and Dr Vincent Hearing (NCI, NIH), respectively, and were used in immunofluorescence experiments at dilutions of 1:500 and 1:100, respectively. Mouse monoclonal antibodies to TRP1 (MEL5) and the endoplasmic reticulum marker BiP were from Signet Laboratories (#8150-01) and StressGen Biotechnologies (#5PA-827), respectively,

and were used at dilutions of 1:50 and 1:200, respectively. Rhodamine-phalloidin (Molecular Probes, #R415) was used at a final concentration of 30 nM. Goat anti-rabbit IgG secondary antibodies were purchased from Molecular Probes (rhodamine, #T-2769; fluorescein, #F-2765; Texas Red, #T-6391). Goat anti-mouse IgG secondary antibodies were from Jackson Immunoresearch (fluorescein, #115-095-146; Texas Red, #115-075-146). All of the double-staining experiments presented in this paper (except those done using rhodamine-phalloidin) were performed using FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse secondary antibodies at dilutions of 1:200 and 1:100, respectively. The Cy5-labeled anti-mouse secondary antibody used in Fig. 8 was purchased from Amersham (# PA45002) and used at a dilution of 1:500.

Western blotting

Brain extracts were prepared by homogenizing individual brains in 0.5 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.5, 0.5 mM EDTA, pH 7.5, 0.5 mM PMSF, and 2 μg/ml leupeptin. To prepare whole cell extracts, confluent T75 flasks were washed with HBSS and incubated for 10 minutes in HBSS containing 1 mM EDTA, pH 7.5, to detach the cells. Cells were collected by centrifugation at 1,200 ***g*** for 5 minutes at 4°C and fully resuspended in HBSS (~300 μl/confluent T75). To this cell suspension, and to the brain homogenate, was added an equal volume of SDS sample buffer (0.03 M Tris-HCl, pH 6.5, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 1 mM EDTA, pH 8.0, and 15% (w/v) sucrose) at 4°C, and the samples were boiled for 5 minutes. After cooling, the samples were triturated four times through a 23 gauge needle to shear DNA. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and processed for ECL-based western blotting (Amersham, #RPN 2108) as described previously (Jung et al., 1996). For quantitation, ECL autoradiograms were scanned using a laser densitometer within the linear response range.

Immunofluorescence

Cells plated on glass coverslips coated with gelatin (0.2%) (Sigma, #G2500) were fixed for 15 minutes at room temperature in freshly-prepared 4% paraformaldehyde (Polysciences, #00380) in PBS or in cytoskeletal stabilization buffer (Conrad et al., 1993), washed with PBS, and blocked for 30 minutes in three changes of PBS containing either 10% fetal calf serum or 10% normal goat serum. Cells were then incubated sequentially for 1 hour each at room temperature with primary and secondary antibodies diluted in blocking buffer containing 0.2% saponin (Sigma, #S7900) for permeabilization (with a washing/blocking step in between). After a brief wash with PBS, the coverslips were mounted on glass slides using Slow Fade antifading mounting medium (Molecular Probes, #S-2828). We note that we obtained staining patterns for myosin V and F-actin that were similar to those obtained using the standard protocol above when cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, treated with sodium borohydride, and permeabilized in 100% acetone at –20°C for 1 minutes, or when cells were fixed and permeabilized by rapid freezing in liquid propane cooled to –140°C, followed by substitution into 99% methanol/1% formaldehyde at –20°C over a period of ~30 minutes (Rocklin et al., 1995). Samples were viewed with a Zeiss Axioplan microscope equiped with the appropriate Zeiss barrier filters and with either a ×63 Phase 3 Plan-Apochromat or a ×100 Plan-Neofluar objective. Images were recorded using either Kodak TMAX black and white film (400 ASA) or Fujichrome color film (1,600 ASA). The confocal images (1 μm sections) shown in Fig. 8 were obtained using a Zeiss LSM 410 confocal microscope.

Special care was taken to eliminate the possibility that the striking colocalization seen for myosin V and TRP1 in our double-stained images was artifactual. First, we found that in samples which were stained with myosin V antibody or TRP1 antibody alone, no fluorescence was evident in the opposing channel, either visually or photographically (at exposures that gave a bright signal in the correct channel). This lack of significant bleed-through was seen not only when fluorescein and Texas Red were paired (which is what was used in all of the myosin V/TRP1 images presented in this paper), but also when fluorescein and rhodamine (whose emission peaks are even closer together) were paired. Second, when we reversed the normal fluorescent moiety used for myosin V and TRP1 (i.e. rhodamine or Texas Red for myosin V and fluorescein for TRP1), the staining patterns obtained corresponded to those of the primary antibodies. Finally, when we crossed the primary/secondary antibody pairs (i.e. goat anti-mouse secondary with rabbit primary and vice versa) we saw no evidence of cross-species reactivity.

Immunoelectron microscopy

For electron microscopic immunocytochemistry, B10BR melanocytes were cultured in 8-well Permanox culture slides (Nunc, #70410). All procedures were carried out at room temperature on a platform rocker. Melanocytes were fixed for 45 minutes in freshly prepared 4% paraformaldehyde in PBS, washed 3 times for 5 minutes each in PBS, and blocked and permeabilized for 1 hour in PBS containing 5% normal goat serum, 0.2% saponin, and 0.02% sodium azide (blocking buffer). Primary antibodies to myosin V (DIL-1) or TRP1 (PEP1) were diluted in blocking buffer and incubated for 1 hour. The samples were washed for 5 minutes in blocking buffer and twice for 5 minutes each in diluted blocking buffer (1% normal goat serum, 0.1% saponin, 0.02% sodium azide in PBS). Goat anti-rabbit IgG coupled to 1.4 nm gold (Nanoprobes, #2004) was diluted 1:133 in blocking buffer and incubation was for 1 hour. The samples were washed 3 times for 5 minutes each in diluted blocking buffer and twice for 5 minutes each in 0.1 M sodium phosphate buffer (PB), pH 6.8, fixed for 30 minutes in 2% glutaraldehyde in PB, washed 3 times in PB, and stored overnight at 4°C. Silver enhancement of the 1.4 nm gold was carried out for 3 minutes according to the protocol described by Burry (1995). Following 2 washes in PB, the cells were post-fixed in 0.2% osmium tetroxide in PB for 30 minutes, dehydrated in graded ethanols and embedded in a thin layer of Epon. Controls, which were carried out simultaneously in 2 wells of the 8-well culture slide, were treated identically except that the primary antibody was non immune rabbit serum or an irrelevant antibody (rabbit polyclonal antibody to *Acanthamoeba* β-hexosaminidase).

Subcellular fractionation

A subcellular fraction that has been shown by electron microscopy to be highly enriched in end-stage melanosomes (Chakraborty et al., 1989; Orlow et al., 1990; Seiji et al., 1963) was obtained from B16-F10 melanocytes by discontinuous sucrose density gradient centrifugation exactly as described by Seiji et al. (1993). The only change from this now standard procedure was the inclusion in the gradient buffer of 1 mM DTT, 1 mM EGTA, pH 8.0, 25 mM KCl, and protease inhibitors (2 μg/ml each of AEBSF, leupeptin, TLPCK, TPCK, chymostatin, pepstatin, and aprotinin). Briefly, B16-F10 cells (Yamada substrain, which are heavily melanized on a constitutive basis; gift of Dr Vincent Hearing, NCI, NIH) were broken by Dounce homogenization in modified gradient buffer, spun at 600 ***g*** for 5 minutes at 4°C, and the supernatant loaded on a discontinuous sucrose density gradient containing steps of 1.0, 1.2, 1.4, 1.55, 1.6, 1.8 and 2.0 M sucrose. Following centrifugation at 100,000 ***g*** for 1 hour at 4°C, the intensely black interface at 1.8/2.0 M sucrose containing end-stage melanosomes was collected and used for western blot analysis and subsequent manipulations (see text).

RESULTS

Antibodies to TRP1 provide reliable markers for melanized melanosomes

The sole final destination of the melanogenic enzyme TRP1, a type 1 membrane glycoprotein, is thought to be the limiting membrane of the melanosome (Hearing and King, 1993;

Vijayasaradhi et al., 1995). To the extent that this is true, TRP1-specific antibodies could provide reliable melanosome markers, which would facilitate efforts to define the relationship between myosin V and this organelle. To investigate the localization of TRP1, we made use of two established TRPI antibodies. PEP1 is a rabbit polyclonal antibody that was generated against the cytoplasmic tail sequence of mouse TRP1 (Jimenez et al., 1989). MEL5 is a mouse monoclonal antibody that was generated against human TRP1 and that recognizes a highly conserved epitope within the catalytic domain of TRP1. Fig. 1A, shows that these two antibodies are specific for TRP1 in western blots of mouse melanocyte cell extracts. Specifically, the only proteins recognized in B16-F10 and B10BR melanocytes by both antibodies were a family of polypeptides ranging in size from ~65 kDa to ~75 kDa, which spans the size range for TRP1 from its unglycosylated to fully glycosylated forms (Fig. 1, lanes 5-8; similar results were obtained with extracts of melan-a cells). Furthermore, these proteins were not seen in extracts from melan-b cells, which are an immortal melanocyte cell line made from mice that are homozygous for a mutation in the TRP1 gene (Fig. 1, lane 9).

To compare these two antibodies, and to determine the extent to which TRP1 colocalizes with end-stage melanosomes, we double-stained melan-a melanocytes with MEL5 and PEP1 and compared the two fluorescent images to each other and to the phase image, which reveals the positions of heavily-melanized, black melanosomes. Fig. 2 shows that: (i) the two TRP1 antibodies give essentially identical images (B and C); and (ii) the distinctive TRP1-positive fluorescent spots generated by both antibodies largely coalign with the black melanosomes evident in the phase image (A). This coalignment can be seen best in thin regions of the cytoplasm photographed at higher magnification (D,E, F). Furthermore, the closely packed melanosomes present within dendrites, and especially those within dendritic tips, correspond to areas of intense TRP1 fluorescence. These results indicate that both MEL5 and PEP1 represent excellent markers for end-stage melanosomes.

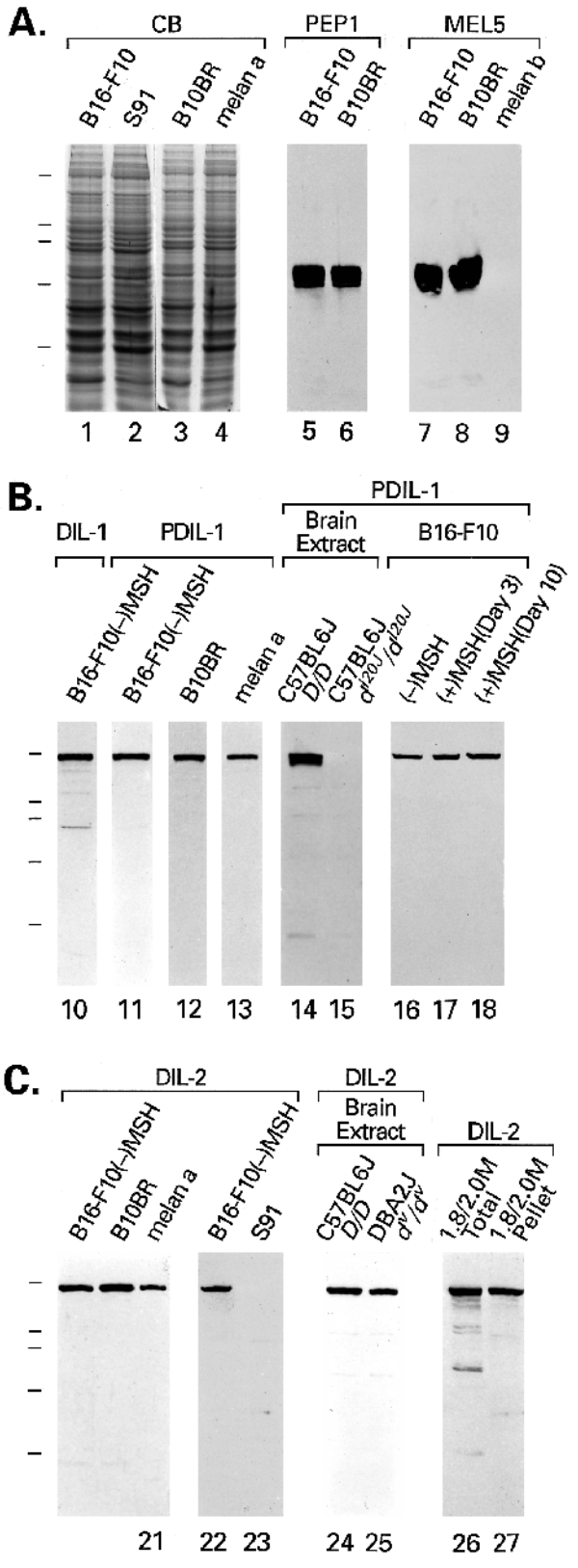

Fig. 1. Immunoblot analyses of the TRP1 antibodies PEP1 and MEL5 (A) and the myosin V antibodies DIL-1 (B) and DIL-2 (C). The melanocyte whole cell extracts that were used throughout are shown in lanes 1-4 (Coomassie blue stain). The nature of each sample and the antibody used to probe it are indicated above each lane. Antibodies were used at the following dilutions: DIL-1, 1:60,000; PDIL-1, 1:1,500; DIL-2, 1:80,000; PEP1, 1:10,000; MEL5, 1:5,000. Whenever lanes are meant to be directly compared, the same amounts of protein were loaded. The molecular mass markers (shown as hash marks) are 200 kDa, 116 kDa, 94 kDa, 67 kDa, and 45 kDa from top to bottom, respectively. Lanes 24 and 25 show blots of brain extracts from *D/D* and d^v/d^v pups, respectively. Laser densitometric scanning indicated that the brains of d^v/d^v mice contain ~50% of the normal level of myosin V (n=3). This result, together with the fact that S91 melanocytes (d^v/d^v) (lane 23) and the brains of d^{120J}/d^{120J} pups (lane 15) are devoid of myosin V, confirm at the protein level the explanation for the tissue-specific expression of the *dilute* phenotype in d^v/d^v animals (based on an analysis of RNA transcripts; Seperack et. al, 1995). Lane 26 shows a blot (probed with DIL-2) of the melanosome-rich subcellular fraction present at the 1.8/2.0 M sucrose interface (see Materials and Methods). This fraction was subsequently diluted threefold with gradient buffer containing 0.2 M sucrose and spun at 40,000 ***g*** for 15 minutes at 4°C to pellet melanosomes. The pellet was resuspended in a volume of gradient buffer equal to that of the original fraction and a sample equal in volume to that in lane 26 was probed with DIL-2 (lane 27). Laser densitometric scanning indicated that ~91% of the myosin V present in the original 1.8/2.0 M fraction pelleted with the melanosomes (n=3).

Antibodies to myosin V are specific for the isoform encoded by the dilute locus

To determine the localization of myosin V in melanocytes, we generated two rabbit polyclonal antibodies against different portions of the mouse myosin V heavy chain isoform encoded by *dilute*. Antibody DIL-1, which was raised against heavy chain residues 1,259-1,853, reacted strongly in western blots of whole cell extracts from unstimulated B16-F10 cells with a single band that corresponds in size to the mouse myosin V heavy chain (~190 kDa), and weakly with several smaller bands (Fig. 1, lane 10). After purification (see Materials and Methods), this antibody (PDIL-1) recognized only the ~190 kDa band in B16-F10 cell extracts (Fig. 1, lane 11). A similar specificity was seen with extracts from B10BR and melan-a cells (Fig. 1, lanes 12 and 13). Proof that this ~190 kDa band is the myosin V heavy chain, and that PDIL-1 is specific for the myosin V isoform encoded by the *dilute* locus, was obtained by probing extracts prepared from the brains of newborn mouse pups that are wild type at the *dilute* locus (lane 14) and pups that are homozygous for the *dilute lethal* allele d^{120J}, which is thought to be a functional null allele for *dilute* (lane 15) (Strobel et al., 1990). PDIL-1 clearly does not recognize other potential myosin V homologs in the null background (at least not those that might be expressed in brain). This result also supports the conclusion that d^{120J} is a true *dilute* null allele. Lanes 16-18 show that the amount of myosin V per cell rises ~2.5-fold when the melanogenic process is stimulated in B16-F10 cells with alpha-melanocyte-stimulating hormone (α MSH) (this increase may represent a significant underestimate, since on average only about two thirds of the cells exhibit significant increases in pigmentation). Antibody DIL-2, which was raised against heavy chain residues 910 to 1,106, was specific as isolated for the ~190 kDa myosin V heavy chain in whole cell extracts of B16-F10 cells, B10BR cells, and melan-a cells (Fig. 1, lanes 19-21). Evidence that DIL-2, like DIL-1, is specific for the myosin V isoform encoded by the *dilute* locus was obtained by probing extracts from the mouse melanoma cell line S91. This cell line was originally derived from a tumor carried by a mouse from the inbred strain DBA/2J, which is homozygous d^{v}/d^{v} (Jenkins et al., 1981). DIL-2 clearly does not recognize other potential myosin V homologs in S91 cells (Fig. 1, lane 23).

To determine whether these myosin V antibodies were also specific at the level of immunofluorescence staining, PDIL-1 and DIL-2 were used to stain primary mouse melanocytes prepared from the dorsal epidermis of newborn pups that were either wild type (*D*/*D*) at the dilute locus (Fig. 3A-C) or homozygous for the *dilute lethal* allele d^{120J} (D-F) (in these primary cultures, which are composed of melanoblasts, melanocytes and fibroblasts, the differentiated melanocytes can be identified based on the presence of black pigment (A and D) and the corresponding fluorescent signal for TRP1 (C and F)). For exposures in which DIL-2 gives bright staining of wild-type melanocytes (B), essentially no fluorescence is visible in mutant melanocytes (E). These and similar results with PDIL-1 (data not shown) indicate that our myosin V antibodies are specific for the myosin V isoform encoded by the *dilute* locus in terms of immunofluorescence staining as well as immunoblotting. This specificity is important since there is evidence that mice express at least one other myosin V heavy chain isoform (*myr6*) (Zhao et al., 1996).

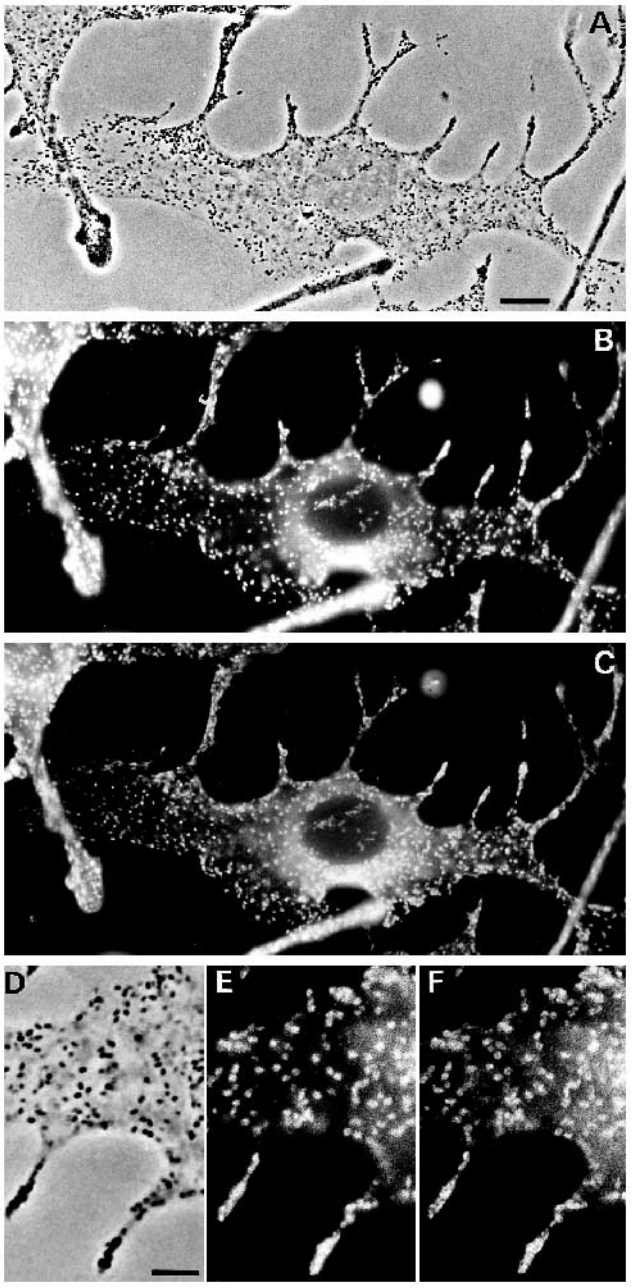

Fig. 2. Colocalization of the TRP1 antibodies MEL5 and PEP1 with b[illegible]nd-stage melanosome[illegible] Shown is a melan-a melanocyte (A) double-stained with MEL5 (B) and PEP1 (C). D, E (MEL5) and F (PEP1) represent a portion of this cell. Bars[illegible] µm (A-C), 5 µm (D-F). The degree of correspondence between the fluorescent spots generated using MEL5 and PEP1 was essentially 100% (n=300). The degree of correspondence of fluorescent spots with black melanosomes in the cell periphery was ~90% for both MEL5 and PEP1 (n=300). Those fluorescent spots that were not black probably correspond to lightly-melanized melanosomes. The degree of correspondence of black, end-stage melanosomes with fluorescent spots was ~94% for both MEL 5 and PEP1.

Myosin V associates with melanosomes during much of the melanogenic process

To investigate the association between myosin V and

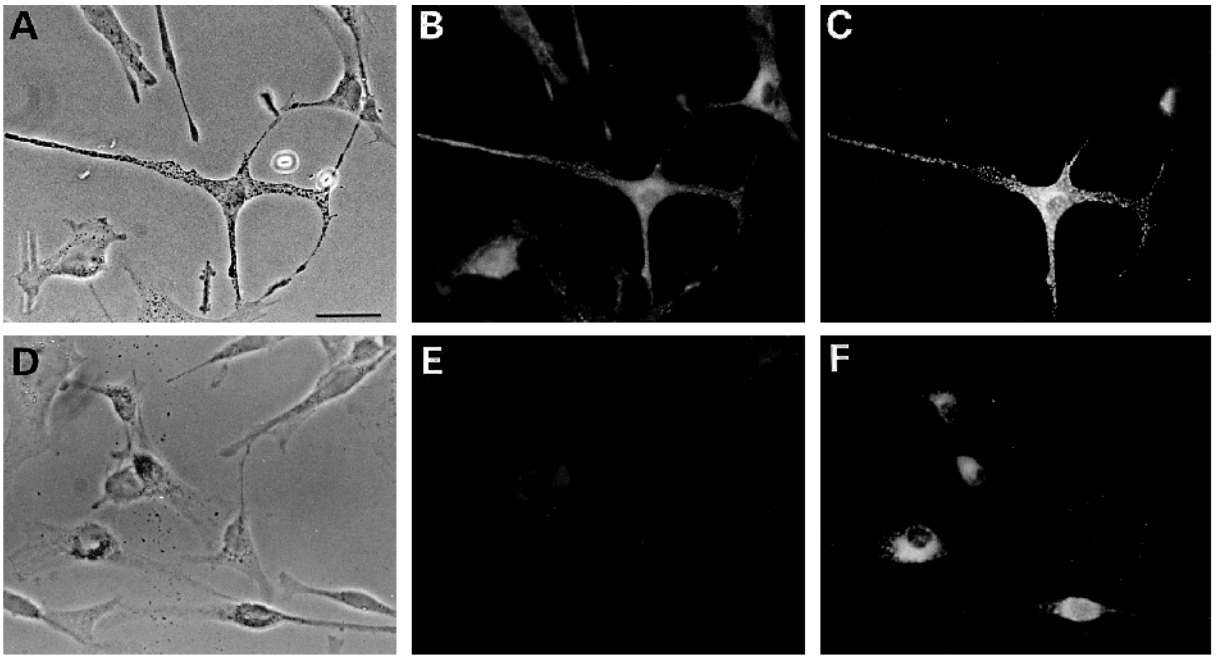

Fig. 3. Control immunofluorescence staining. Primary melanocytes prepared from the skin of wild-type (C57BL/6J *D/D)* (A) and *dilute* null (C57BL/6J d^{l20J}/d^{l20J}) (D) mouse pups were double-stained with the myosin V antibody DIL-2 (B and E) and the TRP1 antibody MEL5 (C and F). Bar, 35 μm. The melanocytes in these mixed primary cultures can be identified based on the presence of black melanosomes (A and D) and the corresponding fluorescence signal for TRP1 (C and F). The exposure time and print development was identical for the images in B and E.

melanosomes throughout the melanogenic process, we used B10-F10 melanocytes, which are unmelanized under basal culture conditions, but become highly melanized after the addition of α MSH to the medium. Fig. 4A shows three B16-F10 cells shortly after the addition of α MSH (~day 2). In the two cells that have not yet responded to the hormone (lower right), TRP1 staining is largely restricted to regions immediately surrounding, and usually to one side of, the nucleus (B). This perinuclear 'cap' of TRP1 staining largely colocalizes with the Golgi-specific marker mannosidase II (data not shown) and most likely corresponds to a small amount of TRP1 undergoing glycosylation within the Golgi (Vijayasaradhi et al., 1995). For the cell that has begun to respond to α MSH (center), TRP1 staining is not only much stronger in the perinuclear region, but is also present as bright punctae radiating out from the center of the cell (B). Many of these bright spots coalign with black granules evident in the phase image (A), indicating that they represent melanosomes undergoing melanization. Fig. 4C shows the staining of these same cells using the myosin V antibody DIL-2. In the two unstimulated cells, myosin V is present as fine punctae distributed throughout the central cytoplasm. In the cell that has begun to respond to α MSH, however, intense spots of myosin V staining are seen over and above the diffuse central staining. Furthermore, most of these myosin V-positive spots coalign exactly with the TRP1-positive spots in B (see the overlaid image in D). Those spots that are positive for both myosin V and TRP1, but which do not coalign with black pigment, probably represent lightly melanized melanosomes, and possibly compartments involved in the delivery of TRP1 to premelanosomes. Fig. 4E and F show the strong colocalization between TRP1 (E) and myosin V (F) in peripheral regions of two B16-F10 melanocytes that are further along in the melanogenic process (~day 4). These images, which were obtained using PDIL-1, also indicate that we see striking colocalization using a second, independent myosin V antibody.

Fig. 5A-C, shows that the colocalization of myosin V with TRP1 and with black melanosomes persists in B16-F10 cells that have become highly melanized and dendritic following extensive treatment with α MSH (~day 6). Careful comparison reveals that within most of the dendrites and dendritic tips in this cell, the fluorescent spots for TRP1 (B) and for myosin V (C) coalign with each other and with end-stage, black melanosomes (A). Fig. 5D-F, show that colocalization of myosin V with TRP1 and with black melanosomes was also seen in B10BR melanocytes, which are heavily melanized and highly dendritic on a constitutive basis. This colocalization is especially evident within the melanosome-rich dendritic tips of these cells. Similar results were obtained with melan-a cells (data not shown). Taken together, the above results suggest that myosin V associates with melanosomes in a specific fashion during a significant portion of this organelle's life cycle, including its movement to the periphery of the cell. Indeed, the coordinate changes in the distributions of TRP1 and myosin V that we see within cells undergoing melanosome biogenesis in response to α MSH strongly support the conclusion that the association between myosin V and melanosomes is physiologically significant.

Immunogold electron microscopy supports the conclusion that myosin v associates with melanosomes

To determine if myosin V actually associates with the limiting membrane of the melanosome (as opposed to being concentrated on structures very near the melanosome), we performed immunolabeling at the electron microscope level. B10BR cells

were fixed exactly as for light microscopy, and then subjected to preembedment labeling with primary antibody. Detection was performed using secondary antibody covalently coupled to 1.4 nm gold, followed by silver enhancement (note that while this approach improves labeling efficiency because of better penetrance relative to larger colloidal gold, the size of the silver enhanced gold grains (arrowheads) is quite variable). Fig. 6 shows a representative example of a melanosome-filled dendrite/dendritic tip stained with myosin V antibody (A, low magnification; C and D, two fields within this extension). In examining these images, it is important to look for the limiting membrane of the melanosome (arrows), which is often a considerable distance away from the electron dense core of the end-stage melanosome. This displacement is seen often in samples fixed for conventional transmission electron microscopy (Fig. 6B; also see, for example, Jimbow et al., 1993), and is very common in samples that have been processed for immunocytochemistry, where the extensive incubations and washes lead to greater membrane displacement, as well as to some loss of melanosomal membrane preservation. This fact is demonstrated in Fig. 6H, which shows melanosomes that were labeled with the TRP1 antibody PEP1, followed by 1.4 nm gold and silver enhancement. The displacements of the heavily-labeled (arrowheads) melanosomal membranes (arrows) from the electron dense cores of the melanosomes seen in this image are very typical, have been reported by many others (see, for example, Orlow et al., 1993a,b; Vijayasaradhi et al., 1995; Zhou et al., 1993), and are probably due in large part to the nature of the melanosome's content, which is that of a highly-insoluble, extensively-crosslinked, osmotically-inert aggregate of melanin polymer. Taking these points into consideration, it is clear in C and D that many of the melanosomes in these two fields are labelled with the myosin V antibody. Fig. 6G shows such labeling at slightly higher magnification. This degree of labeling contrasts sharply with control experiments performed in parallel using either an irrelevant primary antibody (E and F, α *Acanthamoeba* β hexosaminidase) or nonimmune serum (data not shown), where we did not observe labeling of melanosomes. Finally, we note that not all of the gold grains in C and D are

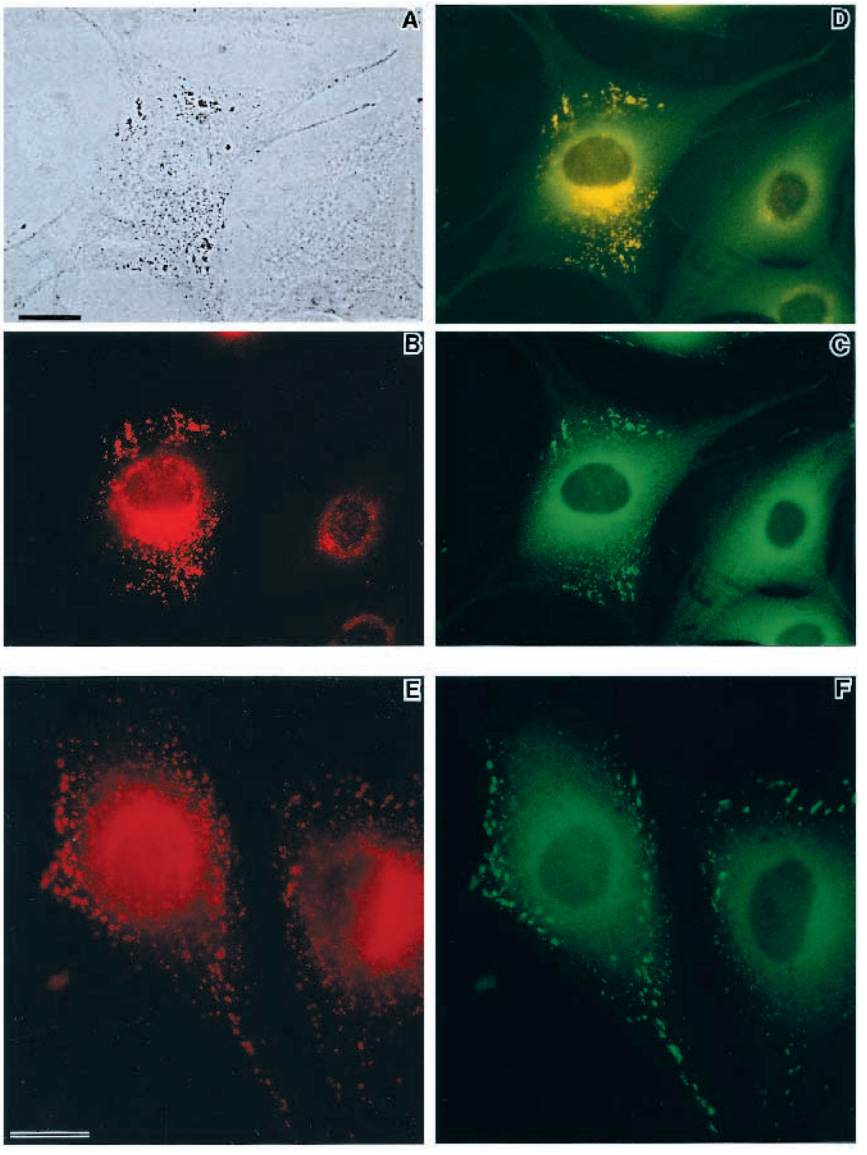

Fig. 4. Colocalization of myosin V with melanosomes in B16-F10 cells that are responding to α MSH. (A) Three B16-F10 melanocytes after ~two days of treatment with α MSH. The cell in the center has begun to respond to the hormone, while the two cells in the lower right corner have not (this variability in response was typical, especially after such a short term exposure to α MSH). These cells were double-stained with the TRP1 antibody MEL5 (B) and the myosin V antibody DIL-2 (C). (D) The overlaid image, which was obtained by capturing on film both fluorophores simultaneously using a triple-band pass filter. Bar, 25 μm. (E and F) Two B16-F10 cells after ~4 days of treatment with α MSH. These cells were double-stained with MEL5 (E) and the myosin V antibody PDIL-1 (F). Bar, 25 μm. We note that we did not observe any striking overlap (using both conventional and confocal microscopy) between the fine punctae of myosin V fluorescence that are so prominent in the central cytoplasm and the staining patterns obtained using markers for either endoplasmic reticulum (BiP, fine punctae widely distributed in a reticular pattern) or medial Golgi (mannosidase II, perinuclear cap), indicating that myosin V is not preferentially concentrated on these membranes.

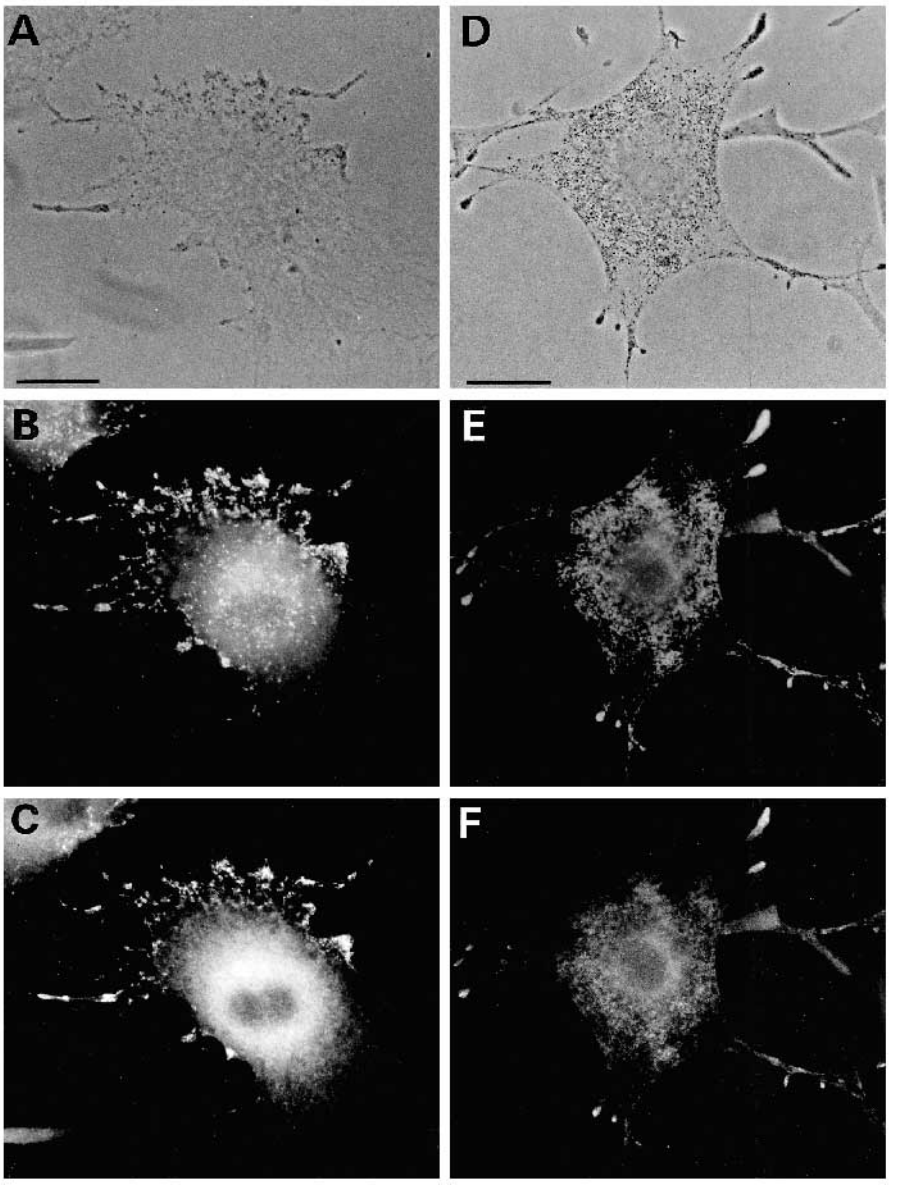

Fig. 5. Colocalization of myosin V with melanosomes in maximally-stimulated B16-F10 cells and in B10BR cells. Shown are a B16-F10 cell that has been maximally stimulated by α MSH (A) and a B10BR cell (D), both of which were double-stained with the TRP1 antibody MEL5 (B and E) and the myosin V antibody DIL-2 (C and F). Bar, 20 μm. The striking colocalization of myosin V with melanosomes was not due to bleed-through of the TRP1 signal (Texas Red here) into the fluorescein (myosin V) channel, based on extensive controls (see Materials and Methods) and on the fact that in cells stained with myosin V antibody alone, striking colocalization was seen between myosin V fluorescence and black melanosomes evident in the light image (data not shown).

melanosome-associated. This is consistent, however, with the diffuse cytoplasmic fluorescence that we see (along with intense melanosome-associated fluorescence) using these myosin V antibodies.

Myosin V is present in a melanosome-rich subcellular fraction

Due to their inherent high density, heavily-melanized end-stage melanosomes can be readily purified from melanocyte extracts by centrifugation through discontinuous sucrose density gradients (Seiji et al., 1963). We found that the material at the 1.8/2.0 M sucrose interface in such gradients, which is intensely black to the eye, and which has been shown to be higly enriched in end-stage melanosomes (Chakraborty et al., 1989; Orlow et al., 1990; Seiji et al., 1963), consistently contained myosin V, based on immunoblot analysis (Fig. 1, lane 26). Moreover, when this fraction was diluted, and the melanosomes pelleted by centrifugation at 40,000 ***g***, greater that 90% of the myosin V present in the original fraction pelleted with the melanosomes (Fig. 1, lane 27). This fact, and the fact that only ~5% of the myosin V in the original fraction pelleted with the melanin polymer when Triton X-100 was added prior to the recentrifugation step (data not shown), further support the conclusion that the myosin V present in the 1.8/2.0 M sucrose fraction is largely melanosome-bound. Together, these results indicate: (i) that the binding of myosin V to the melanosome is of sufficient strength to allow at least some of the myosin to remain bound during the process of subcellular fractionation; and (ii) that these melanosomes may be useful as isolated to test for myosin V-dependent melanosome transport in vitro.

Localization of F-actin suggests that myosin V serves to move melanosomes within dendrites and dendritic tips

Having established that myosin V associates with melanosomes, and given the fact that myosin V is an actin-based mechanoenzyme (Cheney et al., 1993), we sought to

determine the distribution of F-actin within melanocytes, since this might indicate where melanosome movement could be actomyosin V-dependent. To accomplish this, we made use primarily of B10BR cells because they are consistently dendritic and pigmented. Fig. 7B and E shows that the amount and organization of F-actin within these cells, as determined

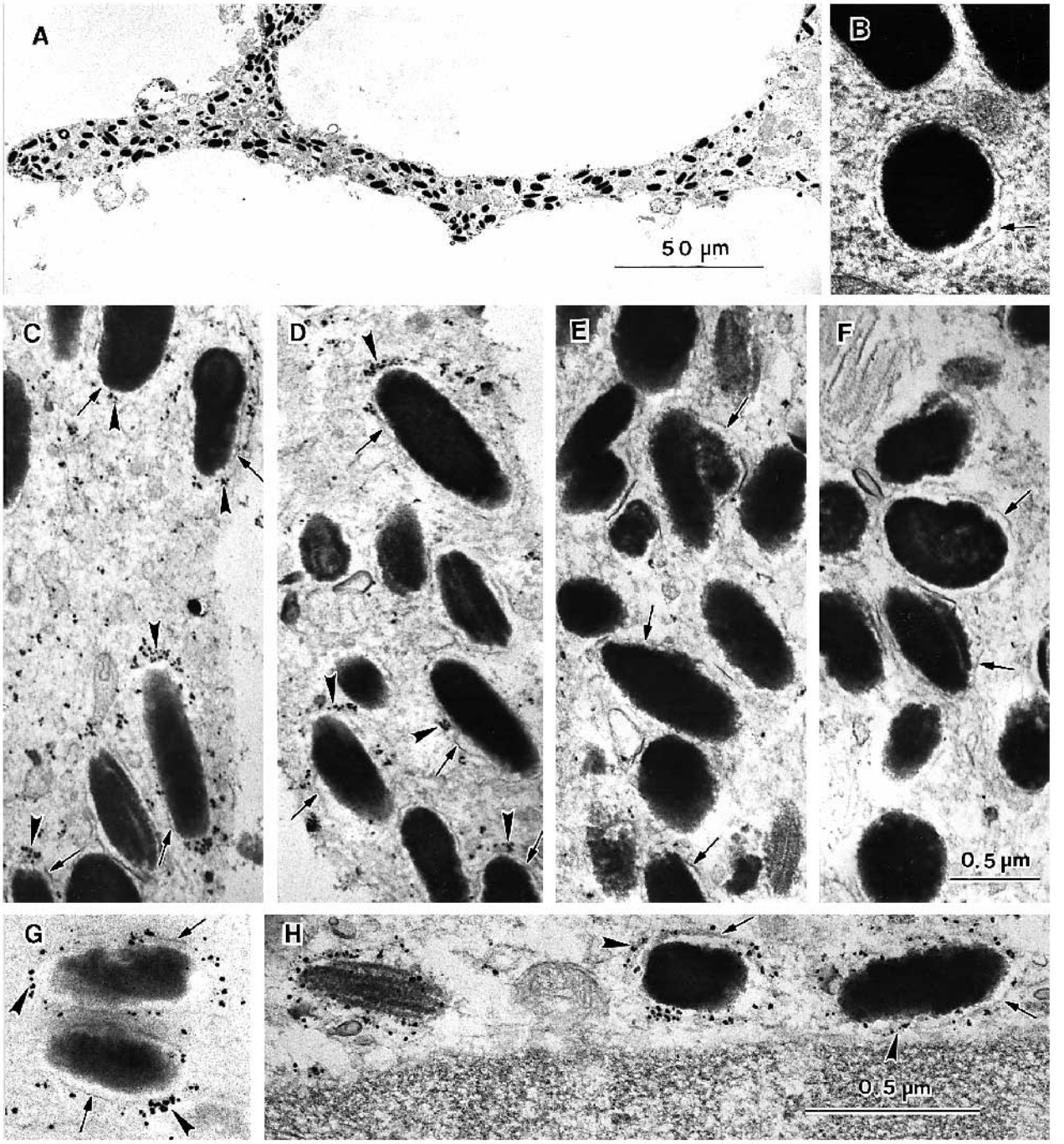

Fig. 6. Immunogold electron microscopy. (A) A dendrite of a B10BR cell that had been stained with myosin V antibody; (C and D) enlargements of two areas within this dendrite. The limiting membranes of the melanosomes, which are not always completely preserved, and which are often a considerable distance away from the dense melanin core, are marked by arrows. Some of the areas in C and D where the melanosome membrane is labeled with myosin V antibody are marked by arrowheads (note that the size of the silver-enchanced gold grains is expected to be somewhat variable; see text). (G) Two melanosomes labeled with myosin V antibody at slightly higher magnification (see magnification bar in H). (E and F) Enlargements of two areas within a dendrite of a B10BR cell that had been stained in parallel using a polyclonal antibody to *Acanthamoeba* β hexosaminidase as an irrelevant antibody. (B) Melanosomes fixed for conventional transmission electron microscopy. Note that the limiting membrane of the melanosome in the center of the image is displaced from the melanin core. (H) Several melanosomes that were labeled with the TRP1 antibody PEP1, which recognizes an integral membrane protein that is specific for the limiting membrane of the melanosome. The imperfectly preserved melanosome membranes can be seen to be displaced from the black cores of the organelles.

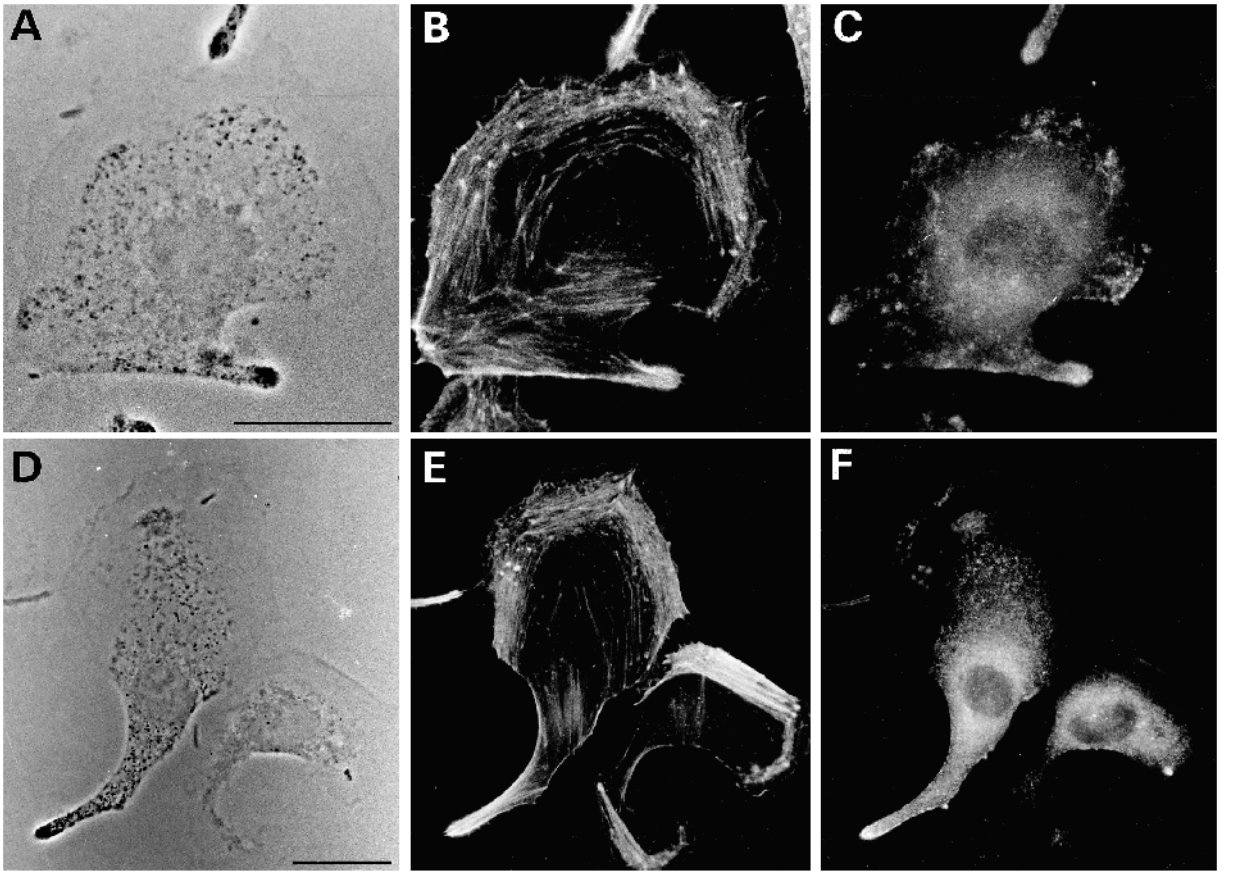

Fig. 7. Localization of F-actin within B10BR melanocytes and colocalization with myosin V. Shown are two B10BR cells (A and D), both of which were double-stained with phalloidin to visualize the distribution of F-actin (B and E) and the myosin V antibody DIL-2 (C and F). The outer edges of the broad, thin, actin-rich, organelle-free lamellae that usually form a rim around much of the cell body are just visible in the phase images (A and D). Bars, 20 μm.

using rhodamine-phalloidin, is region-specific. In the organelle-rich central cytoplasm, F-actin is relatively sparse, being present as a few bundles of variable width, length and orientation. F-actin is present in large amounts, on the other hand, in the broad, thin, organelle-free lamellae (as thick, circumferential bundles), in dendritic extensions (as bundles that appear to run parallel to the long axis of the dendrite throughout its length), and in dendritic tips (as the distal ends of dendritic actin bundles). Confocal microscopy revealed that F-actin is particularly enriched immediately under the plasma membrane throughout dendrites and dendritic tips, and is also present as patches within tips (data not shown). Taken together, then, B10BR melanocytes possess relatively little organized actin centrally, but large amounts of organized actin peripherally within lamellae and dendrites. Of these two types of actin-rich extensions, only dendrites, which are the normal destination for melanosomes, actually contain end-stage melanosomes (see A and D).

These results also suggest that myosin V may contribute to the outward movement of melanosomes within dendrites and dendritic tips. Consistent with this, strong colocalization between F-actin (Fig. 7B and E) and myosin V (Fig. 7C and F) was seen throughout dendrites and dendritic tips. Fig. 8 shows such colocalization within a relatively long, melanosome-rich dendritic extension (A, melanosomes; B, myosin V; C, F-actin). By contrast, the actin-rich, melanosome-free lamellae were essentially devoid of myosin V (Fig. 7C and F). From these results we conclude that the only actin-rich protrusions in which myosin V is concentrated are those involved in the transport of melanosomes to the periphery (i.e. dendrites). We also conclude that myosin V probably does not play a role in the dynamics of those actin-rich surface extensions that are most likely involved in cell motility and shape change (e.g. lamellae).

DISCUSSION

Myosin V and melanosomes

In this study we demonstrated by immunolocalization that myosin V associates with melanosomes in mouse melanocytes. This association, which has also been reported recently by Provance et al. (1996), and in preliminary form by Nascimento et al. (1996), was seen at the light microscope level in all of the melanocyte cell lines we examined, and with two different myosin V antibodies, both of which appear to be absolutely specific at the levels of immunoblotting and immunofluorescence for the myosin V isoform encoded by the *dilute* locus. Furthermore, this association was also seen by immunoelectron microscopy and by subcellular fractionation. The associ-

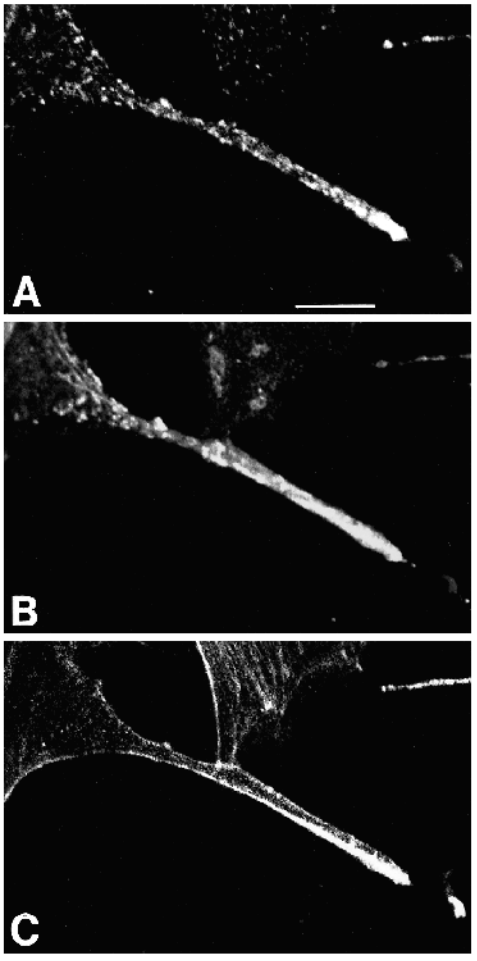

Fig. 8. Colocalization of melansomes, myosin V and F-actin within dendritic extensions. Shown is a confocal image of a relatively long, melanosome-rich dendritic extension that was triple-stained for melanosomes (using MEL5, with a Cy5-labelled anti-mouse secondary antibody) (A), myosin V (using DIL-2, with an FITC-labeled anti-rabbit secondary antibody) (B), and F-actin (using rhodamine-phalloidin) (C). By using confocal microscopy to remove out-of-focus fluorescence, which is very significant in such extensions given their narrow dimensions coupled with their high content of F-actin, myosin V and melanosomes, a significant degree of colocalization between these three markers can be seen. Bar, 12 μm.

ation between myosin V and melanosomes was found not only within completely melanized cells, but also within cells undergoing the process of melanization. Indeed, the coordinate changes in the distributions of the melanosome marker TRP1 and myosin V that were seen within cells undergoing melanosome biogenesis strongly support the conclusion that the association between myosin V and this organelle is physiologically significant. These latter results also suggest that in addition to its association with black, end-stage melanosomes, myosin V may associate with lightly-melanized melanosomes, and possibly with compartments involved in the delivery of TRP1 to premelanosomes.

In some ways our results are similar to those obtained by Espreafico et al. (1992), who observed intense staining for myosin V at the tips of neurites and in the central cytoplasm of cultured hippocampal neurons. This latter fluorescence was especially intense in regions of the Golgi complex (based on partial colocalization with wheat germ agglutinin in frozen tissue sections), which they interpreted as evidence that myosin V associates with Golgi-derived vesicles. While we found that myosin V is not concentrated on the Golgi apparatus itself (see legend to Fig. 4), the large numbers of punctae positive for both TRP1 (a membrane glycoprotein that is processed in, and buds from, the Golgi) and myosin V that we observed shortly after the addition of α MSH suggest that myosin V may indeed associate with Golgi-derived vesicles in melanocytes. Resolution of this question must await, however, the use of an immunoelectron microscopic technique that yields better preservation of cellular fine structure than the preembedment labeling technique employed here.

B16-F10 cells that had become heavily melanized and dendritic following α MSH treatment were found to contain at least two to three times as much myosin V as unstimulated cells. While there could be something special about this additional myosin V in terms of its affinity for the melanosome, it seems more likely that the association of myosin V with this organelle is due to the recruitment of the myosin from a homogenous pool of molecules. This recruitment process may be controlled by post translational modification of myosin V (or the integral and/or peripheral melanosomal membrane protein(s) that are presumably involved in the specific binding of myosin V), and may involve the melanocyte-specific exons identified in the myosin V heavy chain (Seperack et al., 1995). Regarding the fact that unstimulated melanocytes express significant amounts of myosin V, we have found that myosin V (either the *dilute* isoform or the *myr6* isoform) is expressed in every one of the many mouse tissue types that we have examined (Q. Wei and J. A. Hammer, unpublished observations). This fact suggests that type V myosins perform some sort of general or 'housekeeping' function, in addition to cell-type specific functions (e.g. melansome transport?). This general function, which is probably supported by redundant molecules and mechanisms, may be related to some aspect of constitutive intracellular membrane traffic.

Efforts to localize motor proteins on melanosomes within melanocytes from amphibians have been complicated by the fact that in those cells the melanosomes tend to bind irrelevant antibodies as well as antibodies to motor proteins (V. Gelfand and L. Haimo, personal communications). We did not find such a complication in staining mouse melanocytes. For example, we never saw staining of melanosomes using secondary antibodies alone, or using nonimmune serum, antibodies to irrelevant antigens (e.g. *Dictyostelium* myo J and *Acanthamoeba* B-hexosaminidase), or antibodies to a variety of membrane compartments (e.g. mannosidase II and BiP). Furthermore, the black melanosomes that are concentrated in the center of primary melanocytes prepared from *dilute* null mice (which do not express any myosin V; Fig. 1) did not stain with either myosin V antibody (Fig. 3). Finally, myosin V and TRP1 fluorescence strongly colocalize within melan-c melanocytes whose melanosomes are devoid of melanin polymer (data not shown).

Myosin V, melanosomes and the dilute phenotype

Given that myosin V is known to possess actin-dependent mechanochemical activity (Cheney et al., 1993; Wolenski et

al., 1995), our demonstration by immunolocalization that this motor protein associates with melanosomes suggests: (i) that it powers all or some portion of the melanosome's journey from its site of formation in the cell body to its site of intercellular transfer at the dendritic tip; and (ii) that the reduction in coat color intensity seen in *dilute* mice is caused by the absence of this myosin V-dependent melanosome transport system. While such a transport defect is not consistent with the long-standing explanation for coat color dilution, which is that *dilute* melanocytes are adendritic (reviewed by Silvers, 1979), it is supported by one older and two recent studies where cell shape and pigment distribution were analyzed in cultured melanocytes. First, in a study utilizing cultured *D/D* and d^{v}/d^{v} melanocytes (isolated from C57BL/6J and DBA/2J mice, respectively), Koyama and Takeuchi (1981) found: (i) that mutant melanocytes possess essentially normal dendritic arbors; and (ii) that end-stage melanosomes in these cells are highly concentrated in the cell body and essentially excluded from dendrites. In wild-type melanocytes, on the other hand, black melanosomes were found throughout both the cell body and the dendrites. Second, Provance et al. (1996) have recently shown that the results of Koyama and Takeuchi (1981) hold true for d^{v}/d^{v} melanocytes cultured from mice of the same genetic background as the control *D/D* melanocytes (i.e. C57BL/6J). Finally, we have recently shown that the results of Koyama and Takeuchi (1981) also hold true for cultured melanocytes that are homozygous for a true *dilute* null allele (d^{120J}) (Wei et al., 1997; also, see Fig. 3A and D). Furthermore, using an antibody to the membrane tyrosine kinase receptor cKIT to visualize the shape of melanocytes present in epidermal sheets, we have shown that *dilute* melanocytes in situ, like cultured *dilute* melanocytes, are defective primarily in melanosome distribution, not cell shape. These studies (and especially the in situ study) indicate that the clumping of pigment evident in unstained preparations of skin, hair follicles, and Harderian gland from *dilute* mice, which has traditionally been interpreted as evidence that the cells lack dendritic extensions (reviewed by Silvers, 1979), is actually due to a defect in melanosome transport within cells of essentially normal shape. Furthermore, these studies implicate the dendrite as the region of the cell where melanosome transport is myosin V-dependent. Our results with actin staining, as well as the effects of cytochalasin treatment on melanosome distribution in B16 melanocytes (Koyama and Takeuchi, 1980) are consistent with this. Finally, we note that the distribution of F-actin in mutant melanocytes appears to be essentially normal (Lacour et al., 1992; Preston et al., 1987; Wei et al., 1997), indicating that the apparent defect in melanosome transport is not secondary to myosin V-dependent defects in the actin cytoskeleton. This fact contrasts with results showing that the yeast myosin V mutant *myo2-66* exhibits a disorganized actin cytoskeleton in addition to an apparent defect in vesicle transport (Johnston et al., 1991) (although yeast lacking a second myosin V isoform (myo4; Haarer et al., 1994), which also may be involved in intracellular transport (Bobola et al., 1996), have a normal actin cytoskeleton).

Summary

Evidence has been accumulating that type V myosins serve as vesicle/organelle motors (Bobola et al., 1996; Espreafico et al., 1992; Govidan et al., 1995; Johnston et al., 1991; Langford, 1995; Lillie and Brown, 1992, 1994). Our demonstration that myosin V associates with melanosomes in mouse melanocytes adds additional support to this idea. Furthermore, the facts that myosin V is excluded from actin-rich lamellae, that the migration of melanocyte precursors from the neural crest to the skin is normal in *dilute* null mice (Jackson, 1994), and that *dilute* null melanocytes appear to possess relatively normal dendritic shape, all suggest that myosin V does not play a critical role in either cellular motility or the determination of cell shape. These results are consistent with recent studies on the growth cones of SCG neurons, where an association between myosin V and organelles was seen, and where the cytoskeletal architecture of these growth cones and the rate of neurite outgrowth were both found to be normal in SCG neurons isolated from homozygous *dilute lethal* mice (L. Evans, J. A. Hammer, III, and P. C. Bridgman, unpublished observations). These results do not agree, however, with a recent study implicating myosin V in filopodial extension by DRG neurons (Wang et al., 1996). Finally, with regard to the roles of myosins in intracellular motility, we note that in many cases either the motor is known and the organelle is not (Govidan et al., 1995; McGoldrick et al., 1995; Mermall et al., 1994), or the organelle is known and the motor is not (Kachar and Reese, 1988; Kuznetsov et al., 1992; Simon et al., 1995). In this work, however, we know both the motor and the organelle. And while we have yet to demonstrate that myosin V actually moves melanosomes in vivo, this particular motor/organelle association has been documented within a cell type where the absence of the myosin appears to result in a defect in the intracellular transport of the organelle in vivo.

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