Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin to the organelle

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SUMMARY

The peripheral accumulation of melanosomes characteristic of wild-type mouse melanocytes is driven by a cooperative process involving long-range, bidirectional, microtubule-dependent movements coupled to capture and local movement in the actin-rich periphery by myosin Va, the product of the dilute locus. Genetic evidence suggests that Rab27a, the product of the ashen locus, functions with myosin Va in this process. Here we show that ashen melanocytes, like dilute melanocytes, exhibit normal dendritic morphology and melanosome biogenesis, an abnormal accumulation of end-stage melanosomes in the cell center, and rapid, bidirectional, microtubule-dependent melanosome movements between the cell center and the periphery. This phenotype suggests that ashen melanocytes, like dilute melanocytes, are defective in peripheral melanosome capture. Consistent with this, introduction into ashen melanocytes of cDNAs encoding wild-type and GTP-bound versions of Rab27a restores the peripheral accumulation of melanosomes in a microtubule-dependent manner. Conversely, introduction into wild-type melanocytes of the GDP-bound version of Rab27a generates an ashen/dilute phenotype. Rab27a colocalizes with end-stage melanosomes in wild-type cells, and is most concentrated in melanosome-rich dendritic tips, where it also colocalizes with myosin Va. Finally, neither endogenous myosin Va nor an expressed, GFP-tagged, myosin Va tail domain fusion protein colocalize with melanosomes in ashen melanocytes, in contrast to that seen previously in wild-type cells. These results argue that Rab27a serves to enable the myosin Va-dependent capture of melanosomes delivered to the periphery by bidirectional, microtubule-dependent transport, and that it does so by recruiting the myosin to the melanosome surface. We suggest that Rab27a, in its GTP-bound and melanosome-associated form, predominates in the periphery, and that it is this form that recruits the myosin, enabling capture. These results argue that Rab27a serves as a myosin Va ‘receptor’, and add to the growing evidence that Rab GTPases regulate vesicle motors as well as SNARE pairing.

Key words: Rab, Myosin V, Melanosome, Organelle motility, Melanocyte

INTRODUCTION

The pigmentation of mammalian hair and skin is a multistep process involving the synthesis of pigments (melanins) within melanosomes, the specialized, pigment-producing organelles of melanocytes, the long-range transport of fully pigmented melanosomes out the melanocyte’s extensive dendritic arbor, the accumulation of these organelles at dendritic tips, and the intercellular transfer of the organelles from these tips to adjacent follicular or epidermal keratinocytes (Hearing and King, 1993). Genetic evidence suggests that the products of the mouse coat color genes dilute and ashen function together at some point in this pathway (Jackson, 1994; Silvers, 1979). Dilute and ashen mice exhibit identical degrees of coat color dilution, and mice homozygous for both mutations exhibit the same reduction in hair color intensity as the individual mutants (N. G. Copeland and N. A. Jenkins, unpublished observations). Furthermore, the coat color phenotypes of both mutations are suppressed by the semidominant suppressor dsu (Moore et al., 1988). Previous work has shown that dilute encodes the approx. 190 kDa heavy chain of a class V unconventional myosin, myosin Va (Mercer et al., 1991). This myosin colocalizes with black, end-stage melanosomes (Nascimento et al., 1997; Wu et al., 1997; Lambert et al., 1998), and melanocytes isolated from mice homozygous for a functional null allele at dilute (dl20J) exhibit an abnormal distribution of melanosomes, with most being clustered in the cell center (Koyama and Takeuchi, 1981; Provance et al., 1996; Wei et al., 1997).

We have shown that the peripheral accumulation of end-stage melanosomes characteristic of normal melanocytes is driven by a cooperative mechanism in which fast, long-range, bidirectional, microtubule-dependent melanosome movements along the length of dendrites are coupled to myosin Va-dependent capture and local movement within the distal, actin-rich regions of the dendrite (Wu et al., 1998). In dilute melanocytes, where the capture mechanism is disabled, melanosomes become concentrated in the cell center because this is where the bulk of microtubules reside. Long-range, microtubule-based melanosome movements within dendrites continue in the absence of myosin Va, but the bidirectional nature of these movements prevents them from generating a peripheral accumulation of organelles on their own. In wild-type melanocytes, on the other hand, the myosin Va-dependent
interaction of melanosomes with F-actin in the periphery prevents a fraction of the organelles delivered there by centrifugal microtubule-dependent movements from being returned to the cell center by centripetal microtubule-dependent movements, thereby causing their distal accumulation (Wu et al., 1998).

Using positional cloning, RT-PCR analysis and BAC transgene rescue, Wilson et al. have recently shown that ashen encodes Rab27a (Wilson et al., 2000), a largely uncharacterized Rab family member cloned previously from rat megakaryocytes and human melanocytes (Chen et al., 1997; Nagata et al., 1999). Rab GTPases reside on the surface of organelles and vesicles in the endocytic and secretory pathways, where they are thought to play critical roles in the targeting and fusion of these vesicles with their appropriate acceptor membranes by participating in the formation and/or function of SNARE complexes (Martinez and Goud, 1998; Novick and Zerial, 1997). Like other ras-related GTPases, the transition of Rab27a from their active, GTP-bound state to their inactive, GDP-bound state serves as the kinetic control for the processes they regulate. Ashen mice contain an A-to-T transversion in the third base pair of the splice donor site located downstream of exon 4 within the Rab27a gene (Wilson et al., 2000). The aberrant Rab27a transcripts produced as a result of this point mutation are predicted to be nonfunctional since they would generate a truncated protein lacking two domains critical for the formation of the GTP binding pocket. While little is known about the function of Rab27a in melanocytes, ashen melanocytes in culture were shown to resemble dilute melanocytes in that they also exhibit an abnormal accumulation of black melanosomes in the cell center (Wilson et al., 2000).

Here we sought to determine the role that Rab27a plays in melanosome transport and distribution, in part by analyzing the dynamics of end-stage organelles in ashen melanocytes. From these and other experiments, we conclude that Rab27a plays a key role in regulating the motility machinery dictating the distribution of fully differentiated melanosomes, that it does so by enabling the myosin Va-dependent capture of the organelle in the actin-rich periphery, and that this is accomplished by the Rab27a-dependent recruitment of the myosin onto the melanosome surface in a GTP-dependent manner.

**MATERIALS AND METHODS**

**Primary and immortal melanocyte cultures**

Short-term cultures of primary melanocytes were prepared from the skins of newborn mice as described previously (Wu et al., 1998), with the following modifications. Individual full-thickness dorsal skins were trypsinized by floating in 5 ml of trypsin solution (LTI, #25300-054) for 18 hours at 4°C. The epidermis was then separated from the dermis, immersed in 1 ml of TAV medium (Ham’s F10 medium supplemented with 10% horse serum, 2% fetal calf serum, 1% penstrep, 0.1 mM dibutryl cAMP and 85 mM phosphor 12-myristate 13-acetate (Sigma, #P-8139)), chopped into approx. 1 mm \(^2\) pieces, brought to 10 ml with TAV medium, and vigorously triturated. Dissociated cells were collected by filtration through a 100 μm nylon filter (Falcon, #352360) followed by centrifugation for 5 minutes at 200 g, plated on gelatin-coated Lab-Tek glass coverslip chamber slides or #1.5 round coverslips, and maintained in TAV medium. Primary cultures prepared in this way consist mainly of melanocytes and fibroblasts. To eliminate fibroblasts, G418 was added to a final concentration 0.1 mM for 24 hours on approx. day 5 of the culture. The cultures were used for microinjection starting 4 days after removal of G418, and discarded after 3 weeks. Control melanocytes were cultured from C3H/HeSnJ (D/D, ASH/ASH) pups, ashen melanocytes from C3H pups homozgyous for the sole known ashen allele (Lane and Womack, 1979), and dilute melanocytes from C57BL/6J pups homozygous for the dilute null allele d\(^d\)01, which were identified in litters as described previously (Wu et al., 1998). The immortal mouse melanocyte cell line melan-a (D/D, ASH/ASH) was a gift of Dr Dorothy Bennett (St George’s Hospital Medical School, London, UK). The immortal ashen mouse melanocyte cell line melan-ash (D/D, ash/ash) was derived from a primary ashen melanocyte culture as described previously (Wilson et al., 2000).

**Immunofluorescence and video microscopy**

Cells were fixed and processed for antibody staining exactly as described previously (Wu et al., 1997). Microinjected cells were fixed 6-8 hours post-injection. When staining for Rab27a, cells were detergent extracted prior to fixation by incubation for 2 minutes in PHEN buffer (60 mM Pipes, 25 mM Hepes, pH 7.5, 5 mM EGTA, 3% sucrose) containing 0.1% Triton X-100. Samples were viewed using a Zeiss LSM 510 confocal microscope and acquired as single, approx. 1 μm thick optical sections, or a projection of stacked approx. 1 μm sections. Endogenous Rab27a, as well as unfused Rab27a expressed from microinjected plasmid, was visualized using a mouse monoclonal antibody to human Rab27a (Signal Transduction Labs, #R52320) at a dilution of 1:100. Cells expressing FLAG-tagged Rab27a were stained with a 1:200 dilution of the anti-FLAG mouse

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**Fig. 1.** Western blots. (A) Whole cell extract of primary wild-type (C3H) melanocytes probed for Rab27a (molecular mass markers: 200, 116, 97, 66, 55, 38, 31 and 21 kDa, from top to bottom, respectively). (B) Equal loads of whole cell extracts from primary wild-type (C3H) melanocytes (lane 1), primary ashen melanocytes (lane 2), and the immortal ashen melanocyte cell line melan-ash (lane 3) probed for Rab27a.

**Fig. 2.** Phenotype of ashen melanocytes. (A) Brightfield (a-d) and phase-contrast (e-h) image pairs for primary wild-type (C3H) melanocytes (a,e), primary ashen melanocytes (b,f), immortal melan-ash melanocytes (c,g) and primary dilute melanocytes (d,h). Bars, 12 μm (a,c); 11 μm (b,d). (B) Brightfield images (i,d), microtubule distributions (b,e) and F-actin distributions (c,f) in wild-type (a-c) and ashen (d-f) melanocytes. Bars, 15 μm (a); 21 μm (d). (C) Transmission electron micrograph of the cytoplasm of an ashen melanocyte showing melanosomes at all stages of maturation (stages I through IV; see Hearing and King, 1993). Bar, 0.2 μm. (D) Dendrite of an ashen melanocyte (a), and some of the rapid centrifugal (CF) (b) and centripetal (CP) (c) melanosome movements that occurred within the boxed area shown in (a) over a 3 minute interval. Bars, 3 μm (a); 1 μm (c).
monoclonal antibody M5 (Eastman Kodak, #IB13091). Myosin Va was visualized using the rabbit polyclonal antibody DIL-2 (Wu et al., 1997) at dilution of 1:250. Actin and microtubules were visualized using 30 nM Oregon Green 488-labeled phalloidin (Molecular Probes, #0-7466) and a 1:100 dilution of a rat monoclonal antibody to α-tubulin (Accurate Chemical, #MAS 077), respectively. Goat antirabbit IgG-Texas Red conjugate (#075-144), goat antirabbit IgG-FTTC conjugate (#095-144), donkey antismouse IgG-Texas Red conjugate (#075-140), and donkey antirabbit IgG-Texas Red conjugate (#712-075-153) were all purchased from Jackson Immonoresearch and used at a dilution of 1:200. The movements of melanosomes within ashen melanocytes were recorded by time-lapse video microscopy exactly as described previously for dilute melanocytes (Wu et al., 1998; Wu and Hammer, 2000).

**Microinjection**

Microinjection was used to introduce various Rab27a constructs into primary and immortal melanocytes. To identify injected cells, plasmid EGFPCC1, which drives the expression of unfused GFP, was co-injected with the plasmid encoding Rab27a. Purified plasmid DNAs were dissolved in DEPC-treated water at 0.5 mg/ml and centrifuged at 18°C for 30 minutes at 100,000 g to remove particulates. Intracellular microinjection was performed using an Eppendorf microinjection system (model 5171) and Femtotip II microinjection needles (Eppendorf, #930-00-003-5). An injection pressure of 75 psi and an injection time of 0.3 seconds were used.

**DNA constructs and general methods**

A cDNA encoding full-length rat Rab27a (Nagata et al., 1990) was a generous gift of Dr Koh-ichi Nagata (Nagoya, Japan). All non-epitope tagged versions of Rab27a (wild type; T23N (GTP-bound); C219S plus C221S (geranylgeranyl deficient)) were cloned into pcDNA 3.1+ (Invitrogen, #V790-20). Wild-type Rab27a tagged at its N terminus with the FLAG epitope tag was expressed using plasmid pCMV-Tag 2B (Stratagene, #211172). A cDNA encoding full-length rat Rab27a (Nagata et al., 1990) was a generous gift of Dr Koh-ichi Nagata (Nagoya, Japan). All non-epitope tagged versions of Rab27a (wild type; T23N (GTP-bound); C219S plus C221S (geranylgeranyl deficient)) were cloned into pcDNA 3.1+ (Invitrogen, #V790-20). Wild-type Rab27a tagged at its N terminus with the FLAG epitope tag was expressed using plasmid pCMV-Tag 2B (Stratagene, #211172). The T23N and Q78L point mutations were placed in Rab27a using a QuickChange site-directed mutagenesis kit (Stratagene, #200518) and the following oligonucleotides: 5′GACTCTGGATGCTGGAGAAACAGACTGTA-CGTACCAGTAC3′ and its complement for T23N, and 5′CAGTTA-TGGGACACGGGCGGCTTGGAGGACTGCTTACCTCTTCCCTCTACT3′ and its complement for Q78L. The C219S plus C221S double point mutant was created by PCR amplification of the entire coding site-directed mutagenesis kit (Stratagene, #200518) and the following oligonucleotides: 5′GACTCTGGATGCTGGAGAAACAGACTGTA-CGTACCAGTAC3′ and its complement for T23N, and 5′CAGTTA-TGGGACACGGGCGGCTTGGAGGACTGCTTACCTCTTCCCTCTACT3′ and its complement for Q78L. The C219S plus C221S double point mutant was created by PCR amplification of the entire coding sequence of Rab27a using Pfu polymerase (Promega, #M774B) and the following 3′ primer: 5′ATCGAGACCTAATGCGCTTAAAC-CCTTTCCTCCTCCTACT3′. All mutations were confirmed by sequencing. End-stage melanosomes were purified from MNT-1 melanoma cells by discontinuous sucrose density gradient centrifugation (Seiji et al., 1963), with modifications (Wu et al., 1997). The intensely black fraction at the 1.82/2.0 M sucrose interface, which has been shown to be highly enriched in stage IV melanosomes (Chakraborty et al., 1989; Orlow et al., 1993; Seiji et al., 1963), was subjected, along with a whole-cell extract of MNT-1 cells, to protein concentration determination and to Western blot analysis using the anti-Rab27a antibody, followed by laser densitometry, in order to determine the fold enrichment of Rab27a on melanosomes relative to whole cells. Western blotting and other general methods were performed as described previously (Wu et al., 1997, 1998).

**RESULTS**

**Ashen melanocytes**

Ashen melanocytes are devoid of Rab27a

While the majority of Rab27a transcripts in tissues from ashen mice are improperly spliced, some correctly spliced mRNAs are present (Wilson et al., 2000). To estimate the reduction in Rab27a protein levels in ashen melanocytes, we performed Western blots on whole cell extracts prepared from primary melanocytes isolated from C3H+/+ and C3H ash/ash mice, as well as from an immortal melanocyte cell line generated from primary C3H ash/ash melanocytes (Wilson et al., 2000), which we refer to as melan-ash. Fig. 1A shows that a monoclonal antibody directed against human Rab27a detects a single band of a molecular mass consistent with Rab27a in C3H+/+ melanocyte extracts. Furthermore, at an exposure that gave a very strong signal for this band in C3H+/+ melanocyte extracts (Fig. 1B, lane 1), no signal was observed in either C3H ash/ash melanocyte extracts or melan-ash extracts (lanes 2 and 3). We conclude, therefore, that Ashen allele represents a true null allele in melanocytes, and that in the context of the melanocyte, the monoclonal antibody is Rab27a-specific.

Ashen melanocytes exhibit normal melanosome biogenesis, dendritic morphology and cytoskeletal organization, but an abnormal accumulation of melanosomes in the cell center

Wilson et al. recently reported that melan-ash melanocytes exhibit a normal dendritic shape, but an abnormal accumulation of black end-stage melanosomes in the cell center (Wilson et al., 2000). Fig. 2A shows that this phenotype, which we also see when comparing melan-ash cells (Fig. 2Ac,g) to wild-type melanocytes (Fig. 2Aa,e), is identical to that of primary melanocytes cultured from C3H ash/ash mice (Fig. 2Ab,f). Therefore, melan-ash cells faithfully replicate the ashen phenotype as regards the abnormal distribution of black melanosomes within cells of normal shape.

The actin and microtubule cytoskeletons are both required to generate the peripheral accumulation of melanosomes characteristic of wild-type melanocytes (Wu et al., 1998), and at least one rab, rab8, has been shown to influence the organization of these two cytoskeletal elements (Perrin et al., 1996). Fig. 2B shows that ashen melanocytes (Fig. 2Bd-f) exhibit normal distributions of both microtubules and F-actin when compared to wild-type melanocytes (Fig. 2Ba-c), indicating that the central accumulation of melanosomes characteristic of these cells is not a consequence of cytoskeletal disorganization, secondary to loss of Rab27a. While the distribution of end-stage melanosomes is altered in ashen melanocytes, melanosome biogenesis appears normal, as these cells are heavily pigmented and contain melanosomes exhibiting normal maturation and ultrastructure (Fig. 2C).

The phenotype of ashen melanocytes closely resembles that of dilute melanocytes

While the majority of melanosomes in ashen melanocytes are accumulated in the cell center, some are always present within dendritic extensions (see Fig. 2Ab,c,f,g), just as in dilute melanocytes (Fig. 2Ad,h; see also Wei et al., 1997; Wu et al., 1998). In dilute melanocytes, the majority of these peripheral melanosomes are undergoing rapid (approx. 1 μm/second), bidirectional, microtubule-dependent movements between the cell center and the periphery (Wu et al., 1998). Time-lapse imaging (Fig. 2Da) indicates that this is also the case for melanosomes in the periphery of ashen melanocytes. Fig. 2Db,e show some of the more obvious
centrifugal (CF) and centripetal (CP) melanosome movements that occurred over a 3 minute interval within the boxed area of the ashen melanocyte shown in Fig. 2Da. These movements were rapid (approx. 1 μm/second), and quickly halted by addition of nocodazole, indicating that they are microtubule-dependent (data not shown), just as in dilute melanocytes (Wu et al., 1998). While we cannot at present exclude the possibility that some aspects of these microtubule-based melanosome movements are slightly different in ashen melanocytes relative to wild type (e.g. different frequency, persistence), this component of melanosome motility is clearly quite robust in the absence of Rab27a. Therefore, like dilute melanocytes, ashen melanocytes possess an efficient means of moving melanosomes to the periphery, but are incapable of accumulating them there. These observations suggest that ashen melanocytes, like dilute melanocytes, are defective in peripheral melanosome capture, that the capture mechanism requires Rab27a as well as myosin Va, and that Rab27a may serve to enable myosin Va-dependent melanosome capture.
Rab27a

Wild-type and GTP-bound versions of Rab27a, but not the GDP-bound or geranylgeranyl-deficient versions, rescue the distribution of melanosomes in ashen melanocytes

Consistent with the idea that Rab27a enables peripheral melanosome capture, microinjection of a plasmid encoding wild-type Rab27a into melan-ash melanocytes resulted in the restoration of melanosome distribution within approx. 8 hours (Fig. 3Aa-c). Approximately 80% (n=55) of injected ashen melanocytes showed full recovery of peripheral melanosome accumulation. This rescue was observed over a wide range of expression levels, as mutant cells injected with fivefold less plasmid rescued efficiently (Fig. 3Ad-f). Addition of 16 μM nocodazole to the medium 20 minutes after microinjection completely blocked rescue (10 out of 10; data not shown), indicating that the long-range, bidirectional, microtubule-dependent component of melanosome transport is required to deliver melanosomes to the periphery for Rab27a- and myosin Va-dependent capture. Like melan-ash cells, primary ashen melanocytes were also rescued by introduction of wild-type Rab27a (26 out of 34 injected cells) (Fig. 3Ag-i). Finally, ashen melanocytes microinjected with Rab27a Q78L, which should bias the Rab towards its GTP-bound state, also rescued (9 out of 14) (Fig. 3Ba,b), although high-level expression of this version of Rab27a appeared to be somewhat toxic.

In contrast to the wild-type and GTP-bound versions of Rab27a, microinjection of Rab27a T23N, which should bias the Rab towards its GDP-bound state, failed to rescue melanosome distribution in 22 out of 22 injected cells (Fig. 3Bc,d). Furthermore, none of 24 ashen melanocytes were rescued by microinjection of Rab27a in which the two C-terminal cysteines at residue positions 219 and 221, which are the sites of addition of C20 geranylgeranyl groups (Seabra et al., 1995), had been changed to serines (Fig. 3Be,f). Together, these results indicate that Rab27a must be capable of binding GTP and of inserting into membranes in order to rescue ashen melanocytes.

Fig. 4. Dominant-negative phenotype in wild-type melanocytes. Brightfield (a) and phase-contrast (b) images of a primary wild-type melanocyte expressing Rab27a T23N and soluble GFP (c). Bar, 10 μm.

The GDP-bound version of Rab27a generates an ashen/dilute phenotype in wild-type cells

Microinjection of Rab27a T23N into wild-type melanocytes resulted with high frequency (19 of 24 injected cells) in the redistribution of end-stage melanosomes back to the center of the cell (Fig. 4). Generation of this ashen/dilute phenotype was specific to the T23N point mutant (data not shown), and was microtubule-dependent, as addition of nocodazole to cells 20 minutes after microinjection halted the redistribution (8 out of 8 cells). This latter result suggests that the redistribution of melanosomes in Rab27a T23N-injected cells, like the redistribution of melanosomes in wild-type cells transfected with dominant-negative tail constructs of myosin Va (Wu et al., 1998), is due to an uncoupling of the organelles from the peripheral capture mechanism, followed by their subsequent redistribution via microtubule-dependent movements to the cell center, the site of greatest microtubule density.

Rab27a colocalizes with melanosomes and is enriched along with myosin Va in melanosome-rich dendrites and dendritic tips

Low magnification images of wild-type melanocytes stained for Rab27a revealed that the Rab is most concentrated within melanosome-rich dendrites and dendritic tips (Fig. 5Aa,b; see arrowheads). High magnification images revealed a strong correspondence between black, end-stage melanosomes and punctate Rab27a staining (Fig. 5Ac,d; see arrowheads). This Rab signal was real because ashen melanocytes stained in exactly the same fashion gave a negligible signal (Fig. 5Ae,f). Rab27a also colocalized with black melanosomes present in the dendrites of ashen melanocytes that had been rescued by microinjection of unfused Rab27a (Fig. 5Ba,b) and Rab27a with a FLAG epitope tag at its N terminus (Fig. 5Bc,d). Consistent with these results, Rab27a was found to be enriched approx. 800-fold relative to whole cell extracts in a subcellular fraction previously shown to be highly enriched in fully differentiated, stage IV melanosomes (Orlow et al., 1993; Seiji et al., 1963). Finally, wild-type melanocytes double-stained for Rab27a and myosin Va revealed significant colocalization of the two proteins along with black melanosomes, especially within dendritic tips (Fig. 5Ca-c; see arrowheads). Together,
Rab27a, myosin Va and melanosome distribution

These results suggest that the melanosome-associated, and presumably GTP-bound, form of Rab27a predominates in the periphery, and that it is this form that enables myosin Va-dependent melanosome capture.

**Rab27a recruits myosin Va on to the melanosome**

Previous work has shown that endogenous myosin Va colocalizes with black, end-stage melanosomes in wild-type melanocytes (see Figs 3-5 in Wu et al., 1997; also see Nascimento et al., 1997; Lambert et al., 1998). Furthermore, a GFP-tagged myosin Va tail domain fusion protein, when expressed in wild-type cells, was shown to colocalize extensively with black melanosomes, in addition to having a dominant-negative effect on melanosome distribution (see Figs 6-8 in Wu et al., 1998). Fig. 6A shows that the myosin Va present in *ashen* melanocytes exhibits no obvious enrichment...
on the black melanosomes accumulated in the cell center. Furthermore, Fig. 6B shows that the same GFP-tagged myosin Va tail domain fusion protein mentioned above, when expressed in ashen melanocytes, exhibits no obvious enrichment on black melanosomes. These results are consistent with the idea that Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin on to the melanosome surface.

**DISCUSSION**

In this paper we have presented several independent lines of evidence to support our conclusion that Rab27a enables myosin Va-dependent melanosome capture. First, the phenotype of ashen melanocytes, as regards the distribution and dynamics of end-stage melanosomes, is identical to that of dilute melanosomes (Fig. 2), which have been shown to be defective in peripheral melanosome capture (Wu et al., 1998). Second, reintroduction of Rab27a into ashen melanocytes results in the rapid recovery of peripheral melanosome distribution (Fig. 3). Clearly, ashen melanocytes, which already contain myosin Va, only need Rab27a to reinitiate melanosome capture (Fig. 3). Third, overexpression in wild-type melanocytes of Rab27a T23N, which should displace endogenous Rab27a with a version that interacts minimally with effectors, causes end-stage melanosomes in these myosin Va-containing cells to rapidly assume the distribution characteristic of myosin Va null (i.e. capture-deficient) melanocytes (Fig. 4). Fourth, both of the preceding two phenomena are microtubule-dependent. This observation, together with our direct demonstration that ashen melanocytes retain long-range microtubule-dependent movements (Fig. 2), argues that Rab27a enables the myosin Va-dependent capture of melanosomes delivered to the periphery by bidirectional, microtubule-dependent transport. Fifth, Rab27a must be able to bind GTP (i.e. the wild-type and Q78L versions, but not the T23N version) in order to rescue melanosome distribution in ashen melanocytes (Fig. 3). The fact that Rab27a enables myosin Va-dependent melanosome capture only when it is in its ‘on’ state is entirely consistent with the effect that the nucleotide state has on the function of other Rabs (Novick and Zerial, 1997). Sixth, Rab27a colocalizes and copurifies with melanosomes (Fig. 5), which is a prerequisite for it to have an influence on melanosome distribution. Seventh, Rab27a is most highly concentrated in the melanosome-rich distal ends of dendrites (Fig. 5). This is precisely where melanosome capture must be focused to support the biology of mammalian pigmentation. Furthermore, this is where myosin Va and actin, the other two players in the capture event, are also concentrated (Fig. 5; Wu et al., 1997). We conclude, therefore, that the principal function of Rab27a in mouse melanocytes is to enable the myosin Va-dependent capture of melanosomes in the actin-rich periphery. This functional interaction may allow the extent to which melanosomes are accumulated in the periphery and, consequently, the extent to which they are transferred to keratinocytes, to be under the control of signaling pathways regulating the nucleotide state of Rab27a. We propose that this represents an important site of control in mammalian pigmentation.

With regard to the mechanism by which Rab27a enables myosin Va-dependent melanosome capture, we found that the association of both endogenous myosin Va and an expressed myosin Va tail domain fusion protein with the melanosome is Rab27a-dependent (Fig. 6; Wu et al., 1997; Wu et al., 1998). These results are consistent, therefore, with the idea that Rab27a enables capture by recruiting the myosin on to the melanosome surface, i.e. that Rab27a serves as a myosin Va ‘receptor’. Future efforts will be directed at determining whether the interaction between Rab27a and myosin Va is direct or involves an intervening protein (such as the GEF for Rab27a; see below), and whether two small exons (exons D and F; Seperack et al., 1995), which are present in the melanocyte spliced isoform of myosin Va, but not the brain

Fig. 6. Myosin Va localization in ashen melanocytes. Brightfield (a) and fluorescence (b) images of an ashen melanocyte stained for endogenous myosin Va, which was shown previously to colocalize with end-stage melanosomes in wild-type melanocytes (Wu et al., 1997). Bar, 13 μm. Brightfield (c) and fluorescence (d) images of an ashen melanocyte expressing a GFP-tagged myosin Va tail domain fusion protein, which was shown previously to colocalize extensively with black melanosomes in wild-type melanocytes (Wu et al., 1998). Bar, 12 μm. We note that Rab27a remains melanosome-associated in dilute melanocytes. Therefore, while the association of myosin Va with the melanosome is Rab27a-dependent, the converse is not the case.
spliced isoform, and which are required for the myosin to influence the distribution of melanosomes (X. Wu and J. A. Hammer, manuscript in preparation), are required for Rab27a: myosin Va interaction.

The data presented here add to growing evidence that Rab GTPases regulate motor proteins responsible for vesicle motility, as well as the machinery governing vesicle fusion (reviewed in Martinez and Goud, 1998; Novick and Zerial, 1997). For example, the GTP-bound form of Rab6 has recently been shown to interact physically with a novel, kinesin-like protein named Rabkinse-6 (Echard et al., 1998), and Rab5, which regulates membrane traffic into and between early endosomes, has recently been implicated in the regulation of the movement of early endosomes on microtubules (Nielsen et al., 1999). An even more striking parallel with the work presented here can be found in recent studies on the yeast type V myosin Myo2p and the rab GTPase Sec4p. The polarized secretion of vesicles that supports asymmetric bud growth in yeast is driven by the Myo2p-dependent movement of these secretory vesicles on actin cables from mother cell to bud (Pruyne et al., 1998; Schott et al., 1999). Sec4p, which resides on the surface of these vesicles, is thought to link them to the Exocyst, a multiprotein complex that probably mediates fusion of the vesicles with the bud membrane (Guo et al., 1999). As expected, mutants of Myo2p accumulate vesicles in the mother cell, consistent with a cessation of vesicle transport (Goyidan et al., 1995). Interestingly, mutants of Sec2p, which interacts physically with Sec4p and acts as a nucleotide exchange factor or GEF for Sec4p, also accumulate vesicles in the mother cell (Walch-Solimena et al., 1997). These results indicate that secretory vesicle transport requires Sec2p as well as Myo2p, and suggest that Sec2p, GTP-bound Sec4p, or a complex of the two, recruits Myo2p to the secretory vesicle surface. Myosin V function in yeast as well as melanocytes appears, therefore, to be enabled by Rab GTPases and/or their exchange factors.

The biology of mammalian pigmentation requires that melanosomes be concentrated in dendritic tips for efficient transfer to keratinocytes, since only the pigment present in keratinocytes is visible in hair and skin. While the mechanism of intercellular melanosome transfer is unknown, including whether or not a SNARE complex is involved, the process can be viewed, as for the growth of the bud in yeast, as a form of polarized secretion, and melanosomes are in fact considered to be a type of secretory lysosome (Dell’ Angelica et al., 2000). Therefore, the functional interaction between type V myosins and Rab proteins occurs in the context of polarized secretion in both yeast and vertebrate cells. Underlying these functional interactions may be protein complexes on the vesicle surface that contain not only the type V myosin and the Rab, but microtubule motors as well, since both myo2p (Beningo et al., 2000) and myosin Va (Huang et al., 1999) have been shown to interact physically with kinesin family members (reviewed in Brown, 1999; Wu et al., 2000). Such complexes could provide the means to coordinate the machinery regulating vesicle docking and fusion with the machinery governing vesicle movement and positioning.

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