

Involvement of Dynein and Spectrin with Early Melanosome Transport and Melanosomal Protein Trafficking

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Melanosomes are unique membrane-bound organelles specialized for the synthesis and distribution of melanin. Mechanisms involved in the trafficking of proteins to melanosomes and in the transport of mature pigmented melanosomes to the dendrites of melanocytic cells are being characterized, but details about those processes during early stages of melanosome maturation are not well understood. Early melanosomes must remain in the perinuclear area until critical components are assembled. In this study, we characterized the processing of two distinct melanosomal proteins, tyrosinase (TYR) and Pmel17, to elucidate protein processing in early or late steps of the secretory pathway, respectively, and to determine mechanisms underlying the subcellular localization and transport of early melanosomes. We used immunological, biochemical, and molecular approaches to demonstrate that the movement of early melanosomes in the perinuclear area depends primarily on microtubules but not on actin filaments. In contrast, the trafficking of TYR and Pmel17 depends on cytoplasmic dynein and its interaction with the spectrin/ankyrin system, which is involved with the sorting of cargo from the plasma membrane. These results provide important clues toward understanding the processes involved with early events in melanosome formation and transport.

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INTRODUCTION

Melanosomes are unique membrane-bound organelles that are specialized for the synthesis and the distribution of melanin (Hearing, 2000; Kushimoto *et al.*, 2001, 2003). Melanosomes are produced only by melanocytic cells and normally mature from undifferentiated and unpigmented organelles (termed stage I melanosomes) to differentiated and highly pigmented organelles (termed stage IV melanosomes). Melanosomes are often divided into “early” (ie stages I and II) or “late” (ie stages III and IV) melanosomes which lack or contain pigment, respectively. Tyrosinase (TYR) is an enzymatic protein localized in melanosomes that catalyzes the critical rate-limiting step required for melanin synthesis, that is, tyrosine hydroxylation (Hearing and Ekel,

1976; Körner and Pawelek, 1982; Ujvari *et al.*, 2001; Francis *et al.*, 2003). The trafficking of TYR to melanosomes can be affected dramatically by mutations in several melanogenic genes and by changes of intracellular pH that result in oculocutaneous albinism or hypopigmentation, respectively (Halaban *et al.*, 1997, 2002; Oetting *et al.*, 2003; Watabe *et al.*, 2004). Pmel17 (also known as gp100) is a structural protein of melanosomes that is critical to forming the internal fibrillar matrix characteristic of stage II melanosomes (Kushimoto *et al.*, 2001). The trafficking of Pmel17 to melanosomes is rarely disrupted, since it sorts through both the endocytic and the secretory pathways with relatively high efficiency (Valencia *et al.*, 2006; Valencia *et al.*, 2007). Those characteristics make these two melanosomal proteins suitable targets to study distinct routes of protein trafficking to melanosomes and the transport of those organelles from perinuclear areas of melanocytes to their periphery before their transfer to neighboring keratinocytes.

In melanocytes, mature pigmented melanosomes bind to microtubules and undergo actin-dependent transport toward the cell periphery, before their transfer to keratinocytes (Wu *et al.*, 2002). Thus, the intracellular transport of melanosomes is directed by microtubules (composed of α : β -tubulin dimers) and actin filaments (composed of actin monomers). Microtubules act as tracks for the transport of several intracellular organelles, including lysosomes and sorting vesicles (Lambert *et al.*, 1999; Robertson and Allan, 2000). The trafficking of

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Abbreviations: AP, adaptor protein; ASP, agouti-signaling protein; DOPA, L-3,4-dihydroxyphenylalanine; EndoH, endoglycosidase H; ER, endoplasmic reticulum; α MSH, α -melanocyte stimulating hormone; TYR, tyrosinase

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sorting vesicles to their target organelles is controlled by two classes of microtubule-associated motor proteins, kinesins, and cytoplasmic dyneins. Kinesins power the plus-end-directed microtubule-based motility, while cytoplasmic dyneins drive the minus end motility (Vallee *et al.*, 1988; Schnapp and Reese, 1989). Dyneins and kinesins also have well-established roles in retrograde and in anterograde transport of melanosomes (Byers *et al.*, 2000; Hara *et al.*, 2000; Wu and Hammer, 2000; Vancoillie *et al.*, 2000a). A recent study of melanocytic cells in the amphibian *Xenopus laevis* suggested that the distribution of melanosomes in those cells results from competition between the microtubule-based dyneins and the actin-based myosins, in what was termed a "continual tug-of-war" (Gross *et al.*, 2002). In their model, myosin-Va counteracts dynein-mediated motor transport more effectively than kinesin-mediated transport, which results in the eventual localization of mature melanosomes at the periphery of the cells. Recently, there has been confirmation that a similar mechanism is responsible for melanosome transport in retinal pigment epithelial cells (Lopes *et al.*, 2007). Thus, mechanisms underlying the movement of mature pigmented melanosomes are fairly well characterized, but during their early formative stages, melanosomes need to remain in the perinuclear area until important enzymatic and structural components are delivered. In addition to pigmented MNT1 melanoma cells, we have used SK-Mel-28 melanoma cells, which express all known melanosomal proteins yet remain unpigmented and produce only early-stage melanosomes, to characterize events important to the subcellular localization of those organelles. Interestingly, microtubules are essential both for dispersion and for aggregation of melanosomes in zebrafish after stimulation with α -melanocyte stimulating hormone (α MSH), while microfilaments affect only their aggregation (Logan *et al.*, 2006). However, whether α MSH or its antagonist, agouti-signaling protein (ASP), regulates the expression of motor proteins in mammalian melanocytes is unknown.

Spectrin is a cytoskeletal protein that binds simultaneously to integral membrane proteins, cytosolic proteins and certain phospholipids (either directly or through adaptor proteins (APs)) to create a multifunctional scaffold. Spectrin also binds to all major filament systems and links membranes and cytosolic proteins to cytoskeletal elements (reviewed in De Matteis and Morrow, 2000). Current evidence indicates that the spectrin skeleton plays a role in the early secretory pathway at the endoplasmic reticulum (ER)–Golgi interface (Godi *et al.*, 1998). Nevertheless, there is limited evidence for a role of spectrin in post-Golgi trafficking and in the control of endo-lysosomal compartments (Michaely *et al.*, 1999; De Matteis and Morrow, 2000). A recent study revealed that spectrin localizes in melanophores and plays a role in the transport of melanosomes within melanocytic cells of amphibians (Aspengren and Wallin, 2004), but to date, nothing is known about the possible role of the spectrin skeleton in mammalian melanocytes.

SK-Mel-28 melanoma cells are unpigmented due to a disrupted endocytic pathway that severely affects the endosomal trafficking of TYR, but not the trafficking of

Pmel17, since those cells form stage II melanosomes that depend on Pmel17 function (Watabe *et al.*, 2004). Therefore, we used SK-Mel-28 cells as a model to study mechanisms involved in the trafficking of melanosomal proteins and in the localization of early melanosomes. Analysis of the trafficking of TYR and Pmel17 demonstrated that early melanosomes are transported primarily along the microtubule network and may involve the microtubule-based motor dynein. Proteomic and immunofluorescence analysis provide early evidence for the presence of spectrin in early melanosomes of human melanocytes. Further, the primarily central localization of spectrin in melanocytic cells suggests its role in the aggregation rather than the dispersion of melanosomes. In this regard, spectrin may be involved in the linkage of sorting vesicles, which contain either TYR or Pmel17, to melanosomes. DNA microarray analysis showed that key elements involved in the transport of melanosomes, such as Rab27A and dynein, are strongly downregulated by ASP, which suggests that melanosome transport is disrupted by ASP and further inhibits melanogenesis. Thus, this study provides important clues toward understanding the roles and the mechanisms involved in the maturation and transport of early melanosomes.

RESULTS AND DISCUSSION

Motor-related proteins detected in melanosomes at various stages of maturation

Studies of the dynamics of mature melanosomes in pigmented melanocytic cells have been critical to unraveling the roles of important motor components in the intracellular transport of organelles. However, mechanisms involved in the transport of early melanosomes and their accumulation in perinuclear areas of melanocytic cells are unknown. Our recently published global proteomics analysis of melanosomes (Chi *et al.*, 2006) permits a comparison of proteins present in early and/or late melanosomes derived from pigmented MNT-1 cells, with those in early melanosomes derived from unpigmented SK-Mel-28 cells. Motor-related proteins found in early and/or late melanosomes are summarized in Table 1. From the tripartite complex of Rab27A, melanophilin (SLAC2A) and myosin-Va, which connects mature melanosomes to the actin cytoskeleton (Wu *et al.*, 2006), only Rab27A was present in early melanosomes from both types of melanocytic cells. Interestingly, myosin-Va was present only in melanosomes from pigmented MNT-1 cells, whereas melanophilin was not detected in melanosomes from either type of cell.

Similarly, we searched for the microtubule-based motor proteins kinesin and cytoplasmic dynein, which bind the actin-based motor protein myosin-Va for anterograde melanosome transport (Byers *et al.*, 2000; Vancoillie *et al.*, 2000a,b). Kinesin was found only in melanosomes from pigmented MNT-1 cells, whereas dynein light chain proteins were found in melanosomes from both types of melanocytic cells. These results suggest that early melanosomes are transported via a balanced coordination between kinesins and dyneins in melanocytic cells. However, dynein-dependent motor transport may be predominant in early melano-

Table 1. Motor-related proteins found in melanosomes by proteomics analysis

Protein AC/Gene ID	Protein	SK-Mel-28	MNT-1
P51159/RB27A	Rab-27	Stages I and II	All stages
Q9BV36/MLPH	Melanophilin (SLAC2A)	ND	ND
Q9Y4I1/MYO5A	Myosin-Va	ND	Stages II and IV
P33176/KINH	Kinesin heavy chain	ND	Stage II
Q9H193	Kinesin 13A2	ND	Stage IV
Q9NRC3/Q9NRC3	Cytoplasmic dynein heavy chain (fragment)	Stage II	ND
Q14204/DYHC	Dynein heavy chain, cytosolic (DYHC)	Stages I and II	Stages II and IV
P63172/DYLT1	Dynein light chain Tctex-type 1	Stage I	ND
P63167_DYL1	Dynein light chain 1, cytoplasmic	ND	Stages I and IV
Q9Y6G9/DC1L1	Dynein 1 light intermediate chain	ND	Stage IV
Q14203/DYNA	Dynactin-1	ND	Stage IV
Q13561/DCTN2	Dynactin subunit 2, Dynamitin	ND	Stage IV
Q9UQ16/DYN3	Dynamamin 3	ND	Stage IV
Q13813/SPTA2	Spectrin α chain, brain	Stages I and II	Stages II and IV
Q01082/SPTB2	Spectrin β chain, brain 1	Stages I and II	Stages II and IV
Q9H254/SPTN4	Spectrin β chain, brain 3	Stage II	ND
U49691/ANK1	Ankyrin	ND	ND
P58546/MTPN	Myotrophin (protein V1)	Stage II	ND
P12814/ACTN1	α -Actinin-1	Stages I and II	Stage IV
O43707/ACTN4	α -Actinin-4 (F-actin crosslinking protein)	Stages I and II	ND

ND, not detected.
Data obtained from the melanosome proteome database, as reported by Chi *et al.* (2006).

somes from unpigmented SK-Mel-28 cells, which is consistent with their accumulation in the perinuclear area. Recently it has been reported that the dynactin complex binds both dynein and kinesin 2 and is required for plus-end-directed melanosome transport in amphibian melanophores (Deacon *et al.*, 2003). In our study, dynactin 1 (p150^{glued}) and dynamitin (p50), key elements of the dynactin complex, were detected only in mature (stage IV) melanosomes. These results are consistent with the dual role of this complex in mediating two-way organelle traffic.

The spectrin/ankyrin skeleton is thought to bridge integral membrane proteins, cytosolic proteins, and certain phospholipids, to form a versatile adaptor scaffold. Several studies support a growing body of evidence that beyond the Golgi, many if not all other compartments of the secretory and endo/lysosomal pathways have associated skeletons (reviewed in De Matteis and Morrow, 2000). Spectrin has been identified in several lysosomal-related organelles such as chromaffin granules and synaptic vesicles (Zagon *et al.*, 1986; Aunis and Bader, 1988), and more recently, it has been found in melanosomes in amphibians (Aspengren and Wallin, 2004). Our proteomics analysis identified spectrin- α and spectrin- β II in melanosomes from both types of human melanocytic cells (Table 1). Note that spectrin- β IV, which is associated with secretory granules, was only detected in early-stage melanosomes from SK-Mel-28 cells. Interestingly, the AP ankyrin, which provides the primary linkage between the spectrin-

actin network and the plasma membrane, was not detected in any melanosome fractions. However, the cytoplasmic protein myotrophin (V1), which contains three ankyrin repeats and is involved in the regulation of actin polymerization (Taoka *et al.*, 2003), was identified in early melanosomes from SK-Mel-28 cells. Interestingly, two α -actinins were detected only in early-stage melanosomes from unpigmented SK-Mel-28 cells. α -Actinins belong to the spectrin gene superfamily, which represents a diverse group of cytoskeletal proteins that includes the α - and β -spectrins and dystrophins. α -Actinin is an actin-binding protein with multiple roles in different types of cells. In nonmuscle cells, the cytoskeletal isoform of α -actinin is found along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane (Broderick and Winder, 2005). This result further supports an important role for the spectrin skeleton in early melanosomes. The sum of these results suggests that dynein and spectrin are present in early melanosomes and thus may be involved in their subcellular localization in melanocytic cells, along with the Rab27/melanophilin/ myosin-Va motor and the dynactin complex, which is involved in the transport of mature pigmented melanosomes.

Unpigmented melanocytic cells have an altered distribution of kinesin and spectrin

We then compared the distribution of various motor-related proteins (Figure 1), including β -tubulin (as a marker for

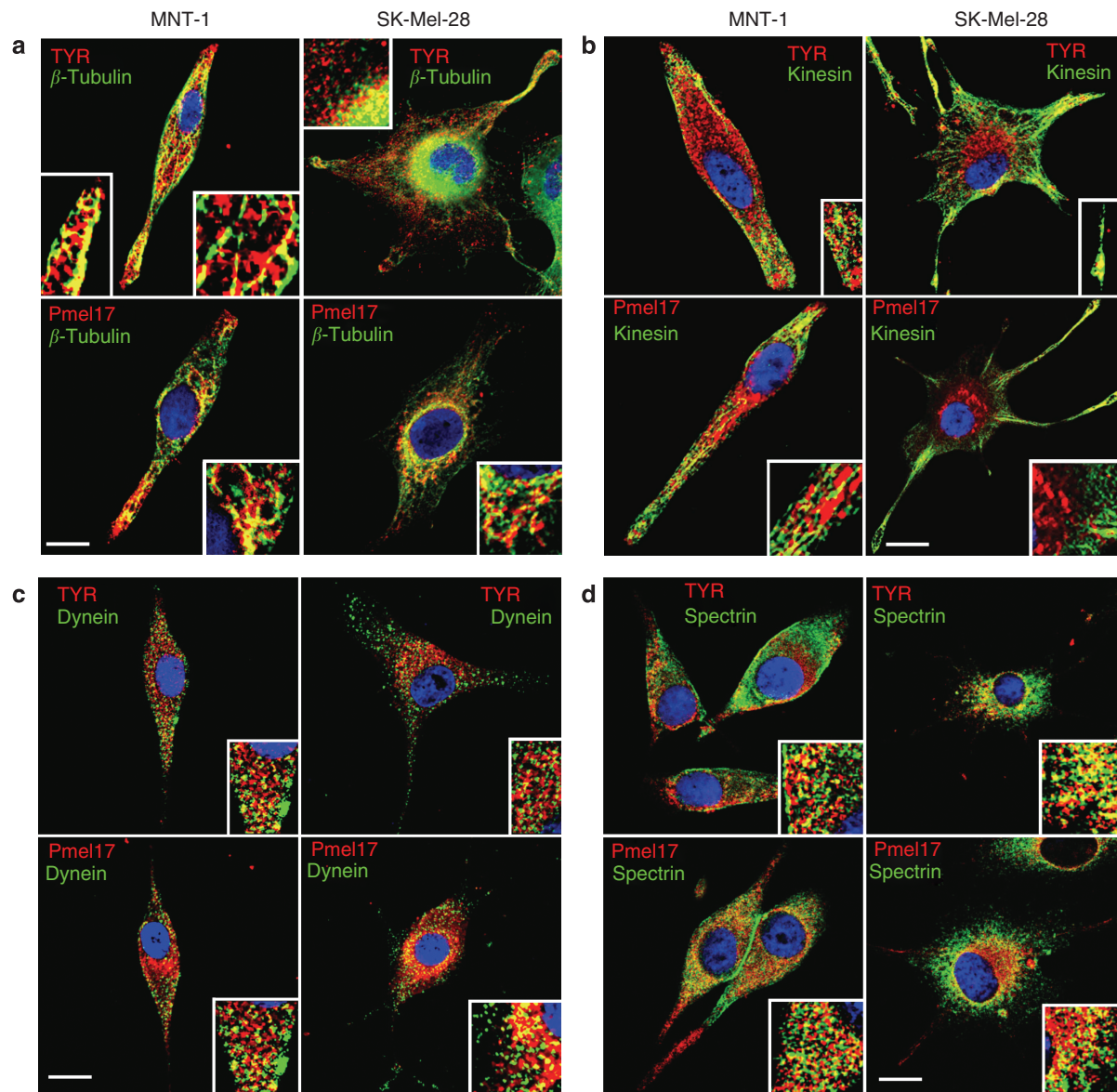


Figure 1. Subcellular localization of TYR and Pmel17 in relation to microtubules, motor proteins and spectrin. MNT-1 cells and SK-Mel-28 cells were fixed and stained with antibodies recognizing TYR (α PEP7h at 1:40) and Pmel17 (α PEP13h at 1:40), both in red, as well as markers for microtubules (a) (β -tubulin at 1:10), kinesin (b, 1:250), dynein (c, 1:250), and spectrin (d, 1:100), all in green. Subcellular localization was examined by confocal microscopy. Insets show higher magnifications of perinuclear or dendrite areas in each panel. Nuclei were counterstained with DAPI (blue). Representative images from two separate experiments are shown for each combination of antibodies. Bar = 10 μ m.

microtubules), kinesins, dyneins, and spectrin, with the localization of TYR and Pmel17 in pigmented MNT-1 cells (left panels in each set) and in unpigmented SK-Mel-28 cells (right panels in each set). In MNT-1 cells, TYR and Pmel17 were evenly distributed throughout the cytoplasm. In contrast, in SK-Mel-28 cells, those proteins showed a predominant perinuclear pattern in all cells, whereas in \sim 15% of SK-Mel-28 cells, a diffuse and less prominent cytoplasmic pattern was observed. TYR or Pmel17 colocalized with organized fibers of microtubules beneath the plasma membrane and especially in dendrites of MNT-1 cells (Figure 1a). However, in SK-Mel-28 cells, the microtubule network was concentrated near the perinuclear area, with thin fibers

extending to the periphery in a disrupted pattern. In SK-Mel-28 cells, β -tubulin colocalized with TYR and Pmel17 mainly in the perinuclear area (inset) but not in the dendrites. Next, we compared the distribution of kinesin and dynein with TYR and Pmel17. Kinesin (Figure 1b) showed a well-defined distribution in the peripheral cytoplasm and in the dendrites, but not in the perinuclear area in pigmented or in unpigmented melanocytic cells. A limited colocalization between kinesin and TYR or Pmel17 was observed at the dendrite tips (insets) in both types of cells. In contrast, dynein (Figure 1c) showed a diffuse granular pattern that colocalized with granules containing either TYR or Pmel17 near the perinuclear area (insets) in both types of melanocytic cells.

These results suggest that dynein-dependent motor transport is predominant in early melanosomes, whereas mature late-stage melanosomes are transported actively to the dendrites by kinesin. This is consistent with the absence of kinesin in early melanosomes from SK-Mel-28 cells.

Analysis of the spectrin distribution also revealed differences between MNT-1 cells and SK-Mel-28 cells (Figure 1d). Spectrin showed a well-defined distribution at the plasma membrane in the central areas of MNT-1 cells but not in their dendrites. Note that the staining of spectrin at the plasma membrane was enhanced at points of cell-cell contact. Surprisingly, there was no staining of spectrin at the plasma membrane of SK-Mel-28 cells. In both types of cells, spectrin in the cytoplasm was distributed mainly in the perinuclear area, where it colocalized with granules containing either TYR or Pmel17, but was not detected in the dendrites of the cells. Taken together, these results show dramatic differences in the distribution of kinesin and spectrin that may be involved in the perinuclear localization of early melanosomes in unpigmented SK-Mel-28 cells. Further, the presence of spectrin in perinuclear areas but not in the dendrites, and its relationship with early melanosomes, suggests a role for spectrin in the aggregation, rather than the dispersion of melanosomes in mammalian melanocytes.

Monitoring of early and late trafficking of melanosomal proteins in the secretory pathway

Protein and vesicle trafficking in the secretory pathway can be separated into early events (for trafficking within the ER-Golgi) and late events (for trafficking from the TGN to target organelles). To evaluate these processing events independently, we characterized the trafficking of TYR and Pmel17 in SK-Mel-28 cells. First, we compared the processing of TYR and Pmel17 using metabolic pulse-chase labeling (Figure 2a). In MNT-1 cells (left panel), α PEP7h recognized TYR as a ~72 kDa band at 0 hour of chase that was slowly glycosylated to ~78 kDa after 6 hours of chase; the intensity of that band was reduced by 53% after 48 hours of chase. Similarly, α PEP13h recognized Pmel17 in MNT-1 cells as a single ~95 kDa band at 0 minute of chase, then as a ~115 kDa band that appeared after 15 minutes of chase. Note that the intensity of Pmel17 was reduced by 47% after 3 hours of chase. In contrast, in SK-Mel-28 cells (right panel), TYR and Pmel17 seemed to be processed correctly at early

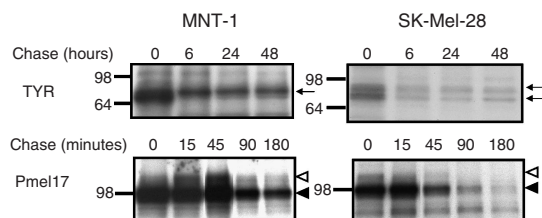


Figure 2. Processing and ultrastructural localization of TYR and Pmel17 in melanoma cells. MNT-1 and SK-Mel-28 cells were [³⁵S] metabolically labeled for 30 minutes, and were chased for times indicated, after which samples were immunoprecipitated with α PEP7h or α PEP13h, as noted. Immunoreactive bands were visualized by autoradiography. Molecular weight markers are indicated on the left in kDa.

chase times, but their stability was severely reduced when compared with MNT-1 cells, by 83 and 90%, respectively.

Next, we used electron microscopy to determine the localization of TYR in MNT-1 cells and in SK-Mel-28 cells (Figure 3). Cells were stained with or without L-3, 4-dihydroxyphenylalanine (DOPA), which is converted into electron-dense DOPA-melanin by TYR, allowing the distribution of DOPA melanin to be assessed. In MNT-1 cells, melanosomes from different stages of maturation, especially pigmented ones, are distributed randomly throughout the cytoplasm. DOPA staining was mainly observed in stage II and stage III melanosomes. In some cases, Golgi structures showed intense DOPA staining (arrowheads). In unstained SK-Mel-28 cells, stage II melanosomes were observed scattered throughout the cytoplasm and near the plasma membrane, which is evidence that Pmel17 was processed correctly. Interestingly, DOPA staining revealed a fine granular staining pattern inside several organelles, such as multivesicular bodies (MVB; asterisks). Note the fine granular

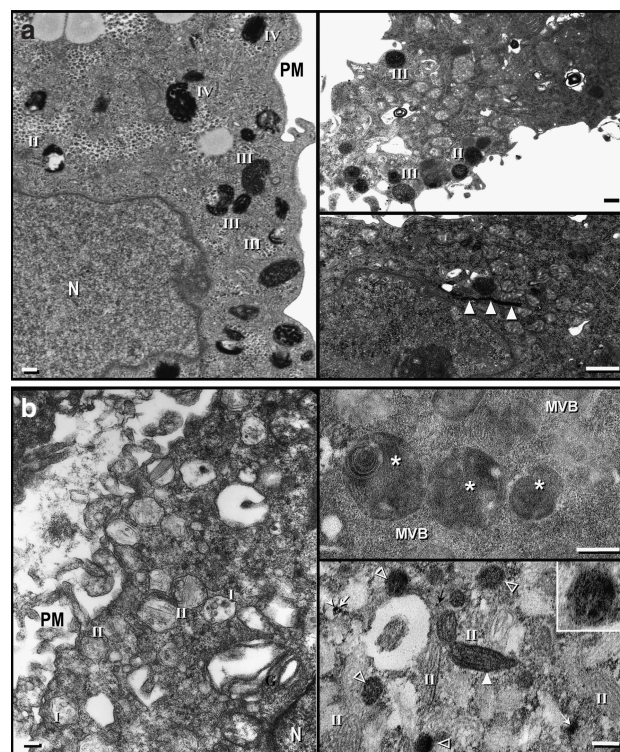


Figure 3. Electron micrographs. Electron micrographs showing the predominant amounts of late-stage (III and IV) melanosomes in MNT-1 cells, and stage II over stage I melanosomes in SK-Mel-28 cells. (a) MNT-1 cells treated without (left panels) or with (right panels) DOPA to demonstrate the presence of functional tyrosinase inside stage II and III melanosomes (top panels), and occasionally within the Golgi (bottom right panel; arrowheads). (b) SK-Mel-28 cells treated with DOPA to demonstrate the presence of functional tyrosinase inside multivesicular bodies (top right panel; asterisks), or inside other intracellular organelles such as stage II melanosomes (bottom right panel, closed arrowheads), rounded vesicles (open arrowheads), or small vesicles (arrows). Inset shows a high-power image of a DOPA-positive vesicle without visible fibrils. Note that most stage II melanosomes are not stained with DOPA. Roman numbers indicate melanosome stages. PM, plasma membrane; N, nucleus; MVB, multivesicular body.

DOPA staining in vesicular organelles (open arrowheads) and very few stage II melanosomes (dark arrowheads). Also note that the DOPA melanin pattern inside vesicular organelles has a diffuse and less organized pattern compared with stage II melanosomes (inset, bottom right). This result indicates that the small amount of TYR sorted from the TGN in unpigmented SK-Mel-28 cells is mostly misdirected to MVB, rather than being sorted to melanosomes, which probably results in its rapid degradation. In contrast, Pmel17 is sorted and incorporated correctly into early melanosomes in SK-Mel-28 cells.

Taken together, we conclude that because of its longer processing time in the ER–Golgi compartment, TYR is a good target to study melanosome protein trafficking in the early secretory pathway. In contrast, the rapid and uninterrupted trafficking of Pmel17 makes it a suitable marker to analyze vesicle sorting and early melanosome transport at late stages of the secretory pathway.

Microtubules are responsible for anterograde trafficking in the early secretory pathway

Bidirectional traffic from the ER–Golgi intermediate compartment to the ER and Golgi depends on intact microtubules (Ben-Tekaya *et al.*, 2005). Because it is the ER–Golgi intermediate compartment to *cis*-Golgi step that requires intact microtubules, we tested the processing and trafficking of TYR and Pmel17 in cells treated with or without nocodazole, an agent that disrupts microtubule function and disrupts the Golgi complex (Salas *et al.*, 1986). SK-Mel-28 cells were metabolically pulse-labeled and were then incubated with nocodazole and chased for various times. TYR and Pmel17 were immunoprecipitated with α PEP7h and α PEP13h antibodies, respectively (Figure 4a). In nocodazole-treated SK-Mel-28 cells, TYR and Pmel17 seemed to be processed normally, but a much weaker mature Pmel17 band (open arrow) was noted compared with untreated cells (compare with Figure 2a). We then examined the sensitivity of TYR to endoglycosidase H (EndoH), an enzyme which removes high-mannose and hybrid-type carbohydrates (Toyofuku *et al.*, 2001; Costin *et al.*, 2003), to evaluate whether nocodazole affected TYR processing through the Golgi (Figure 4b). TYR was only partially sensitive to EndoH after 3 hours of chase and that was associated with a slight increase in stability after 24 hours of chase in untreated SK-Mel-28 cells. In contrast, TYR was completely sensitive to EndoH at all chase times in nocodazole-treated cells. To further confirm those results, lysates of SK-Mel-28 cells treated or untreated with nocodazole were digested with or without EndoH and analyzed by immunoblotting (Figure 4c). In untreated SK-Mel-28 cells, TYR had EndoH-sensitive (S) and resistant (R) bands as expected, but in nocodazole-treated SK-Mel-28 cells, TYR was again fully sensitive to EndoH digestion. These results suggest that nocodazole alters anterograde trafficking of TYR, because it affects its maturation in the Golgi.

Since pH neutralizing agents, such as bafilomycin, improve the sorting of TYR in SK-Mel-28 cells (Watabe *et al.*, 2004), we evaluated whether TYR can be rescued by

combined treatment with nocodazole and bafilomycin (Figure 4c, right panel). TYR remained EndoH-sensitive despite the presence of a weak EndoH-resistant band. The sum of these results suggests that the anterograde transport and processing of TYR in the ER–Golgi compartment depends on microtubules. The effect on protein processing is probably due to arrested movement through the Golgi, preventing access to glycosylation enzymes, as reported previously (Presley *et al.*, 1997).

Actin filaments are critical for sorting in the early secretory pathway

To further evaluate the role of actin filaments in melanosome transport, we characterized the intracellular distribution of F-actin in MNT-1 cells and in SK-Mel-28 cells (Figure 5a). Dual immunofluorescence revealed that actin filaments were distributed mainly at the cell periphery, with fibers observed across the pigmented MNT-1 cells (arrow). In contrast, two distinct patterns of actin filaments were observed in unpigmented SK-Mel-28 cells. One pattern showed actin fibers crossing the cell in several directions (arrows), while the other pattern showed a predominantly granular pattern in some areas (arrowheads). Colocalization of F-actin with TYR was scattered in both types of melanocytic cells near the perinuclear area and also in the dendrite tips of MNT-1 cells. Thus, our results suggest that actin filaments may not be critical for TYR trafficking in the early secretory pathway.

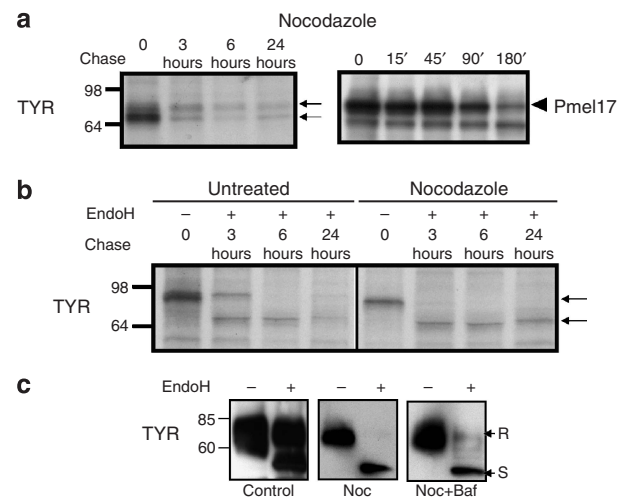


Figure 4. TYR and Pmel17 trafficking depends on microtubules. (a and b) SK-Mel-28 cells were pretreated with 10 mg/ml nocodazole for 3 hours and then were metabolically labeled with [35 S] for 30 minutes. The cells were then chased for the times indicated and were immunoprecipitated with antibodies against TYR or Pmel17, as noted. (b) Immunoprecipitates from untreated or nocodazole-treated samples collected after 0 hour chase were digested with EndoH where noted. (c) SK-Mel-28 cells were left untreated or were treated with nocodazole (Noc) or nocodazole plus bafilomycin A (Baf), for 3 hours before harvest. Cell extracts were then digested with or without EndoH, and TYR was detected by Western blot analysis. Arrows indicate EndoH resistant (“R”) and sensitive (“S”) bands. Immunoreactive bands for metabolic labeling were visualized by autoradiography. Molecular weight markers are indicated on the left in kDa.

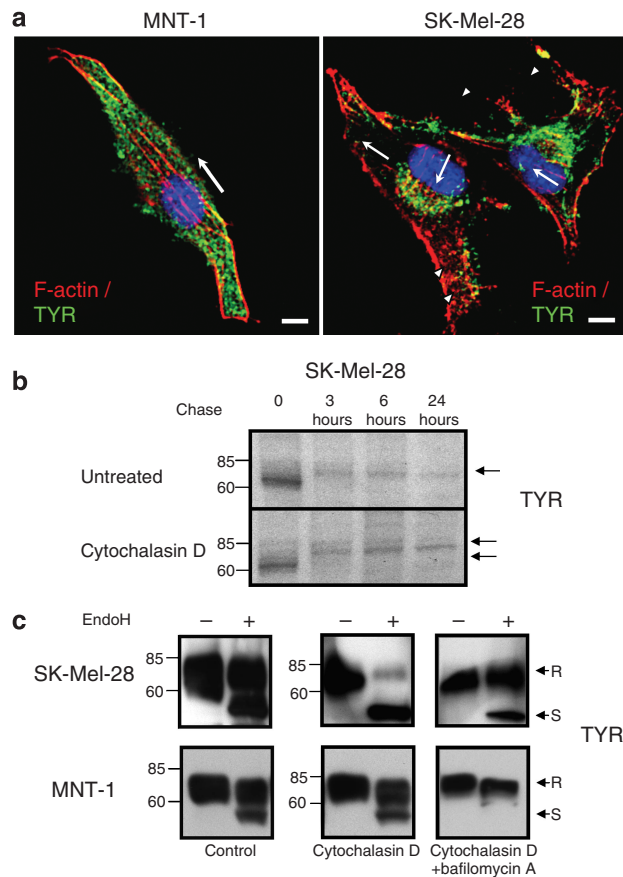


Figure 5. Effect of organellar pH on the trafficking of TYR. (a) Cells were fixed and stained with antibodies recognizing TYR (α PEP7h at 1:40; green) and actin filaments (phalloidin at 1:10; red). Nuclei were counterstained with DAPI (blue). Fluorescence images were collected by confocal microscopy. Bar = 10 μ m (b) Cells were [35 S] metabolically labeled for 30 minutes and were treated with cytochalasin D. Immunoreactive bands were visualized by autoradiography. (c) Extracts were collected from cells treated or untreated with either cytochalasin D or cytochalasin D and bafilomycin A for 3 hours before harvesting. Cell extracts were then digested with or without EndoH, and TYR was detected by Western blot analysis. Bands resistant or sensitive to EndoH digestion are indicated as "R" and "S", respectively. Molecular weight markers are indicated on the left in kDa.

Actin filaments are responsible for the intracellular localization of vacuolar type proton ATPases (Vitavska *et al.*, 2003; Chen *et al.*, 2004), and for maintaining the morphology of Golgi cisternae (Lazaro-Dieguez *et al.*, 2006). We then evaluated whether treatment with cytochalasin D affected the sensitivity of TYR to EndoH digestion, using immunoblotting analysis both in SK-Mel-28 cells and in MNT-1 cells (Figure 5c). In cytochalasin D-treated cells, TYR was either mostly sensitive (SK-Mel-28 cells) or mostly resistant (MNT-1 cells) to EndoH digestion, despite the apparently unaltered size of the protein. Cytochalasin D is known to produce severe swelling of the Golgi cisternae, which leads to a completely disorganized structure and an increase of intra-Golgi pH (Lazaro-Dieguez *et al.*, 2006). To rule out the possibility that TYR formed previously is affecting the appearance of EndoH-resistant bands, we treated cells for

3 hours with cytochalasin D and bafilomycin A1, an agent known to improve TYR stability and processing in the Golgi of melanoma cells (Watabe *et al.*, 2004) (Figure 5c, right). In SK-Mel-28 cells, TYR processing was rescued producing a strong EndoH-resistant band. Consistently, a severe reduction in the intensity of the lower band sensitive to EndoH was observed in MNT-1 cells.

The sum of these results suggests that anterograde trafficking of melanosomal proteins, such as TYR, in the early secretory pathway depends both on pH and on microtubules. In contrast, actin filaments are mainly associated with the maintenance of Golgi morphology and in retrograde transport at this early stage of the secretory pathway (Valderrama *et al.*, 2001).

TYR and Pmel17 are sorted directly to melanosomes in SK-Mel-28 cells

It is well known that TYR follows an indirect sorting pathway through endosomes to reach melanosomes (Watabe *et al.*, 2004), whereas Pmel17 follows both direct and indirect pathways to get to those organelles (Valencia *et al.*, 2006). Since the endocytic pathway is severely disrupted in SK-Mel-28 cells, we hypothesized that TYR and Pmel17 are sorted to melanosomes through a common pathway, probably one that is mediated by AP complexes. To test that hypothesis, we characterized the presence and distribution of AP complexes in SK-Mel-28 cells (Figure 6). First, we isolated several subcellular compartments using a combination of sucrose and iodixonadol gradients and analyzed them by immunoblotting. In the plasma membrane, TYR and Pmel17 were detected in small quantities compared with total cell extracts (Figure 6a). Furthermore, MART1, α -adaptin (AP2) and clathrin were detected in the plasma membrane as well. The enrichment of integrin 5α , a plasma membrane marker, supports the purity of that fraction.

Our proteomics analysis identified only the α - and μ -subunits of AP2 in early melanosomes from SK-Mel-28 cells (Chi *et al.*, 2006). Immunoblot analysis of early melanosomes confirmed that AP2 had the strongest signal in those organelles, followed by very weak bands for AP1 and AP3 (Figure 6b). Note the presence of AP1, AP2, and AP3 in endosomes and clathrin-coated vesicle fractions, which were used as positive controls. Next, we evaluated the distribution of TYR and Pmel17 with AP1, AP2, and/or AP3 in SK-Mel-28 cells using dual immunofluorescence (Figure 6c). TYR and Pmel17 were mainly localized in the perinuclear area, although both proteins were present in the dendrites of SK-Mel-28 cells as well. Colocalization of TYR and Pmel17 with AP1 and AP3 was strong in the perinuclear area, probably as a result of their accumulation in the ER-Golgi compartment. In contrast, Pmel17 showed a wider cytoplasmic colocalization with AP2 than with TYR inside granular structures. These results validate our proteomics study and suggest a major role for AP2 in the trafficking of melanosomal proteins to early melanosomes. Thus, from these results, we conclude that in unpigmented SK-Mel-28 cells, TYR and Pmel17 are sorted through the plasma membrane to early melanosomes in vesicles containing AP2.

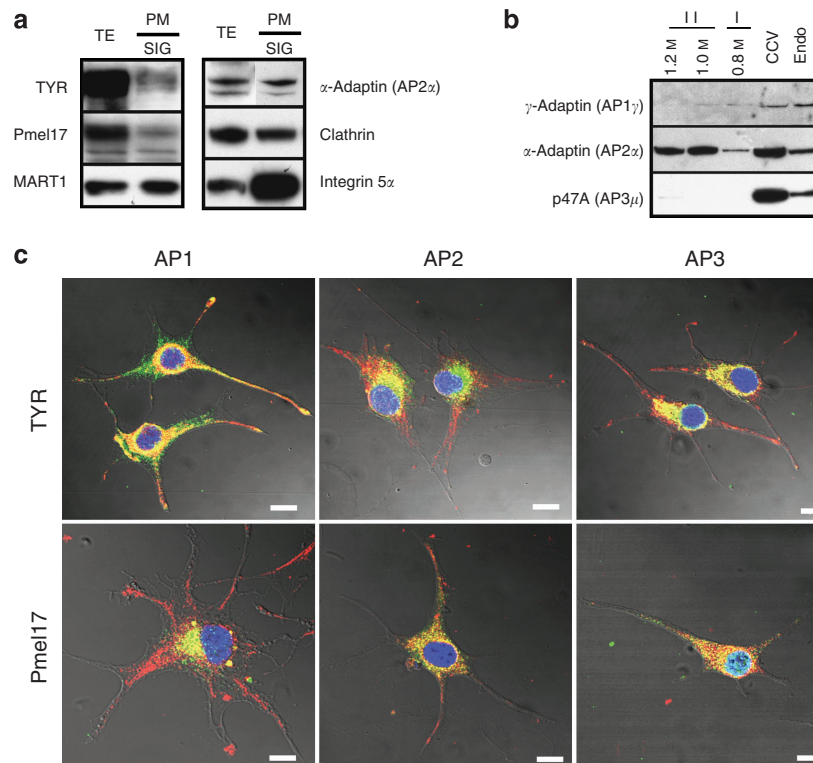


Figure 6. Localization of TYR and Pmel17 in SK-Mel-28 cells. (a) Plasma membrane proteins were recovered by SIG purification and were analyzed by immunoblotting. TYR, Pmel17, and MART-1 were detected in purified fractions. Integrin 4 α was used as a positive control for plasma membrane proteins. Note the presence of AP2 and clathrin in the preparations. (b) Early melanosomes, clathrin-coated vesicles, and endosomes were isolated using sucrose gradients and were analyzed with antibodies against AP1 γ , AP2 α , and AP3 μ . Experiments were performed twice with comparable results. All samples were processed in parallel. (c) Cells were fixed and double stained for tyrosinase or Pmel17 (red) and AP1 γ , AP2 α , or AP3 μ (green), as indicated. Representative images for each combination are shown. Bar = 10 μ m.

Physiological regulation of motor protein expression

Since mature pigmented melanosomes are transported to the tips of dendrites in melanocytic cells, we hypothesized that hormones regulating pigmentation, such as α MSH and/or its physiological antagonist ASP, may regulate the expression of motor proteins and thus affect that process. We tested that hypothesis by evaluating the effects of α MSH and ASP on melan-a melanocytes, which have been previously used to characterize hormonal regulation of pigmentation (Furumura *et al.*, 1996; Sakai *et al.*, 1997; Abdel-Malek *et al.*, 2001). We performed DNA microarray analysis of melanocytes treated for 3 days, either with α MSH or with ASP. The results obtained regarding expression of motor-related proteins are summarized in Table 2.

From the complex of RAB27A, melanophilin, and myosin-Va, DNA microarray analysis revealed that only Rab27A was downregulated significantly by ASP. This is early evidence that expression of Rab27 is regulated by pigmentary hormones. Surprisingly, dyneins and ARP1, major components of the macromolecular complex of dynactin (Schnapp and Reese, 1989), were also significantly downregulated by ASP, while Dnai2 was upregulated by MSH. Note that dyneins and Rab27A are present in early melanosomes from SK-Mel-28 cells by proteomic analysis (Table 2). No elements of the spectrin skeleton were regulated significantly either by

α MSH or by ASP. The DNA microarray analysis also showed that several components of the microtubule anterograde transport machinery, including tubulin α 4A (Tuba4a), tubulin-folding cofactor E, and kinesin family members 21A and 21B, were downregulated by ASP, except for kinesin family member 15, which was upregulated by α MSH. In an attempt to validate the dramatic effects observed for ASP on melan-A cells, MNT-1 cells and SK-Mel-28 cells were treated with ASP or not treated for 3 days. Cell lysates were then prepared and levels of several motor proteins were analyzed by immunoblotting (Figure 7). In SK-Mel-28 cells (Figure 7a), ASP downregulated expression of Rab27, dynein, and kinesin proteins. No change was observed for tubulin or spectrin. In contrast, in MNT-1 cells (Figure 7b), ASP downregulated the key motor protein dynein, whereas no change was observed in other motor proteins, including Rab 27. In both melanoma cell lines, TYR was downregulated by ASP, although this was more evident in SK-Mel-28 cells. These results are consistent with the reduction in TYR activity previously reported for ASP (Furumura *et al.*, 1996). The reason for the differences observed among melanoma cells is not clear. One could argue that the high pigmentation level of MNT-1 cells may have played a role in the reduced effect of ASP. However, to date there has been no report of the effects of ASP on human melanoma cells. In any case, ASP consistently reduced the RNA and protein levels of at least one

key motor protein, dynein, as well as the key melanin producing enzyme, TYR.

Taken together, these results suggest that the expression of key motor proteins is physiologically regulated by α MSH and/or by ASP in melanocytic cells. α MSH promoted the expression of proteins related to the anterograde transport of melanosomes. These results also show that ASP disrupts both pigmentation and melanosome transport by down-regulating key motor proteins such as dynein.

Table 2. Gene expression profiles of motor proteins after treatment of melan-a melanocytes with α MSH or ASP

Expression difference		
MSH	ASP	Gene name
NS	-1.9	RAB27A, member RAS oncogene family (Rab27a)
NS	-1.5	ARP1 actin-related protein 1 homolog B (yeast) (Actr1b)
1.5	-1.5	Dynein, axonemal, intermediate polypeptide 2 (Dnai2)
NS	-1.9	Dynein, axonemal, intermediate chain 1 (Dnaic1)
1.5	-1.5	Tubulin, α 4 (Tuba4)
NS	-1.6	Tubulin-specific chaperone e (Tbce)
-1.6	NS	Kinesin family member 15 (Kif15)
NS	-1.6	Kinesin family member 21A (Kif21a)
NS	-1.5	Kinesin family member 21B (Kif21b)

ASP, agouti-signaling protein; α MSH, α -melanocyte stimulating hormone; NS, not significant. Statistically significant values ($P < 0.01$ considering six analyses) are reported for genes differentially regulated by at least 1.5-fold, either by ASP or by α MSH, in melan-a melanocytes after 3 days of treatment. ($P > 0.01$ or < 1.5 -fold).

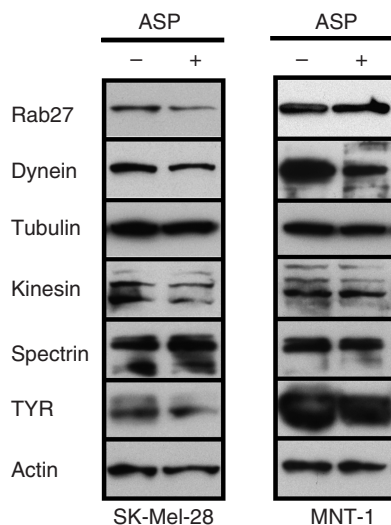


Figure 7. Protein expression profile of motor proteins after ASP treatment of human melanoma cells. SK-Mel-28 cells and MNT-1 cells were treated for 3 days with or without ASP (1:500). New medium was added daily, which contained either sterile water or ASP. Cell extracts were analyzed for protein levels of motor proteins and melanosomal proteins as noted. β -Actin was used as a loading control. Images represent two different experiments.

Final considerations

Genetic and biochemical studies have identified several coat-color mutations in mice that affect the transport of melanosomes (ie *ashen*, *leaden* and *dilute*) (Wu and Hammer, 2000). All three of those genes affect actin-based rather than microtubule-based melanosome motility. In fact, melanosomes still undergo rapid bidirectional, microtubule-dependent movements between the center and the periphery of melanocytes in those mutant cells (Barral and Seabra, 2004). The trafficking and processing of proteins that influence melanin synthesis (e.g. tyrosinase) and the structure (e.g. Pmel17) of melanosomes appear not to be affected by those mutations. It should also be noted that early melanosomes need to remain in the perinuclear area, until delivery of enzymatic and structural components required for their function is complete. Thus, a wide range of regulatory factors and mechanisms remain to be identified that control melanosome maturation in the perinuclear region of melanocytic cells and prevent their dispersion to the dendrites of the cells until they are sufficiently mature. A schematic summarizing these processes and the findings of this study is presented in Figure 8.

Our recently published proteomics analysis (Chi *et al.*, 2006) allowed us to compare early and late melanosomes in pigmented and unpigmented melanocytic cells. Microtubule-dependent intracellular transport is mediated by motor proteins of the kinesin and of the cytoplasmic dynein superfamilies (Chen *et al.*, 2005). Interestingly, dynein is the common motor protein found in early melanosomes, not kinesin. Dynein has a well-established role in the localization and movement of diverse types of cargo, including late endosomes and lysosomes (Valetti *et al.*, 1999). Dynein also plays a significant role as an early endosomal motor and it contributes directly to endosomal fusion, particularly in receptor sorting (Driskell *et al.*, 2007). Further, the confirmation of spectrin to be present in human melanosomes is significant. Our results suggest that spectrin is involved in the aggregation of early melanosomes, but not in their dispersion, due to its centralized distribution (not in dendrites) in melanocytic cells. It is possible that in amphibian melanophores, spectrin may be involved in both processes (Aspengren and Wallin, 2004). Interestingly, the spectrin/ankyrin skeleton is involved in cargo selection and in the interaction with clathrin-coated pits in endocytosis, which tether either microfilaments or microtubules (De Matteis and Morrow, 2000). Therefore, the intracellular distribution of spectrin and the relationship with pigment granules in melanocytic cells suggests a role for the spectrin skeleton to deliver cargo vesicles to target organelles.

Our results showing that movement of early melanosomes is dominated by dyneins and spectrin is consistent with the ongoing maturation process these organelles undergo. These results are also consistent with the fact that only pigmented "mature" melanosomes bind to microtubules for transport to the cell periphery, before their transfer to keratinocytes. In fact, early melanosomes need to be "anchored" in the perinuclear area to receive sorting vesicles containing critical melanosomal proteins needed for their maturation and

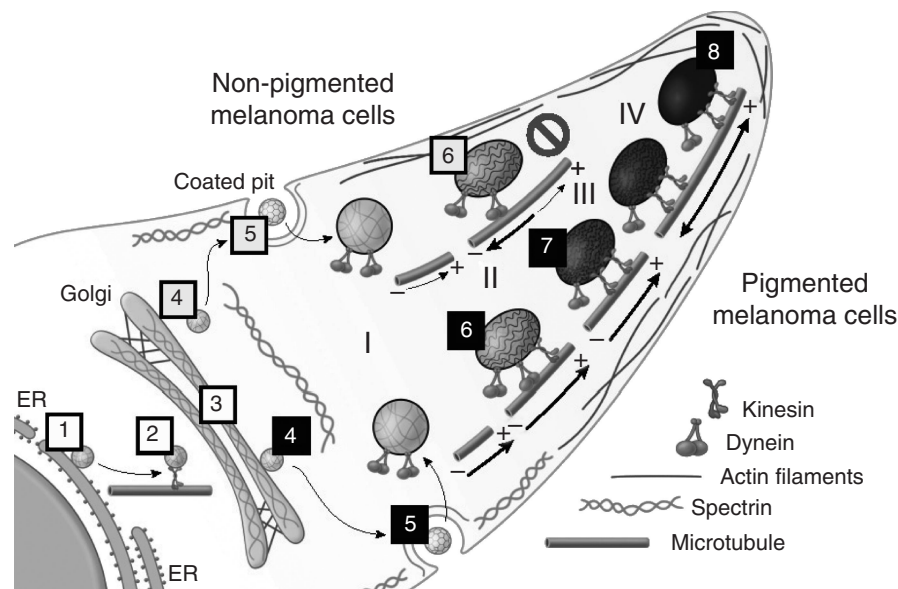


Figure 8. Anterograde movement of sorting vesicles and transport of melanosomes in unpigmented (top) and in pigmented (bottom) melanoma cells. Vesicles containing melanosomal proteins bud from the ER (1). These vesicles are then moved forward by dynein/dynactin along microtubules to the *cis*-Golgi (2). In the Golgi, the spectrin mesh stabilizes the different arriving vesicles and continues the anterograde transport (3). Note the presence of actin filaments at both ends of the Golgi cisternae providing support for this compartment and probably interacting with spectrin. At the TGN (4), sorting vesicles containing spectrin-like mosaics are delivered to downstream compartments in conjunction with other motor and budding systems (only delivery to the plasma membrane is shown to simplify the scheme). A new vesicle is then internalized (5) and directed to stage I melanosomes (6). The presence of dyneins in stage I and II melanosomes possibly favors its accumulation in the central area of the cell. Note that the spectrin-like mosaics in early melanosomes may help to stabilize the organelle and interact with either spectrin-adaptor proteins or some actin filaments (De Matteis and Morrow, 2000). In late melanosomes, the presence of kinesins promotes the transport of these organelles to the cell periphery using microtubules (7). Finally, stage IV melanosomes are transferred to actin filaments for secretion (8). Note the major structural difference between unpigmented SK-Mel-28 cells (top) and pigmented MNT-1 cells (bottom) is the lack of spectrin in the plasma membrane.

eventually for pigment biosynthesis. Our hypothesis is supported by the fact that intermediate filaments may physically hinder organelles in place by making transport more difficult for molecular motors *in vivo* (Kural *et al.*, 2007). Apparently, microtubule-mediated transport of early and late melanosomes may be regulated by the relative amount of two Rab proteins, Rab7 and Rab27a (Jordens *et al.*, 2006).

Physiological regulation of melanosome transport by α MSH has been reported previously in amphibian melanophores (Deacon *et al.*, 2003). In mammalian cells, murine melanocytes represent the best model to evaluate the effects of α MSH and its inverse agonist counterpart, ASP. Therefore, our results further reveal that the expression of motor proteins can indeed be physiologically regulated by those pigmentary hormones. The results obtained with α MSH are consistent with its role in stimulating pigmentation. The results obtained with ASP are very interesting, and in this regard, it has been reported that ASP decreases pigmentation. The role of ASP to downregulate the expression of genes encoding melanosomal proteins (Sakai *et al.*, 1997) or decrease melanosome maturation (Suzuki *et al.*, 1997) in normal melanocytes has been previously shown. However, our results further indicate that ASP also disrupts the transport of early melanosomes. These results are consistent with the current view that overexpression of ASP promotes the de-differentiation of melanocytes (Sviderskaya *et al.*, 2001).

Finally, processing of TYR, but not Pmel17, to melanosomes is affected by genetic alterations in the endocytic pathway, as observed in Hermansky-Pudlak Syndrome (reviewed in Huizing *et al.*, 2001). This suggests the existence of alternative pathways at later steps of the secretory pathway to reach melanosomes. Using SK-Mel-28 cells as a model, we have confirmed that small amounts of TYR and Pmel17 sort through the plasma membrane to reach their intracellular targets. Further, the presence of AP2 in melanosomes, which is also found in the plasma membrane, is consistent with that finding. However, sorting of TYR to melanosomes is less efficient than Pmel17, using that pathway. Apparently, TYR uses AP1 and AP3 to sort through the endocytic pathway (Theos *et al.*, 2005). These results are also consistent with our recent report that Pmel17 uses AP2 and AP1 to reach melanosomes in pigmented melanoma cells or in unpigmented melanocytic cells (Valencia *et al.*, 2006). Our results further complement these studies, since it evaluates the sorting of TYR via an alternate pathway, which does not seem to be used in pigmented melanoma cells.

MATERIALS AND METHODS

Cell cultures

SK-Mel-28 unpigmented human melanoma cells were cultured in minimum essential medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS), as described previously (Watabe *et al.*, 2004). MNT-1 pigmented human melanoma cells were

cultured in DMEM (GIBCO) containing 20% FBS, as described previously (Kushimoto *et al.*, 2001). Melan-a melanocytes, derived from C57BL/6J nonagouti black mice (gift from Professor Dorothy C. Bennett, St George's Hospital Medical School, London, UK) were cultured at 37°C with 5% CO₂ in RPMI 1640 medium (GIBCO) containing 5% FBS (Atlanta Biologicals, Norcross, GA), 7.5% sodium bicarbonate (GIBCO), 200 nM 2-o-tetradecanoylphorbol-13-acetate (Sigma, St Louis, MO), 100 μM 2-mercaptoethanol (Sigma), penicillin and streptomycin (GIBCO), and L-glutamine (GIBCO) at pH 7.2. Where noted, melan-a melanocytes were cultured for 3 days with or without 100 nM αMSH (Sigma) or 1 nM ASP, as previously described (Sakai *et al.*, 1997). All experiments were performed in compliance with guidelines of the National Institutes of Health.

Antibodies and reagents

αPEP7h and αPEP13h antibodies were generated in rabbits against synthetic peptides corresponding to the carboxyl termini of human TYR and Pmel17, respectively, as previously described (Jiménez *et al.*, 1991; Kobayashi *et al.*, 1994). mAbs against kinesin, spectrin, cytoplasmic dynein, and β-actin were purchased from AbCam (Cambridge, UK). Other antibodies used to localize subcellular organelles and cytoskeletal components were as follows: calnexin (ER) from BD Transduction Laboratories (Lexington, KY), vacuolar type proton ATPase and β-tubulin (microtubules) from Santa Cruz Biotechnology (Santa Cruz, CA), and rhodamine phalloidin (actin filaments) from Molecular Probes (Eugene, OR). Bafilomycin and brefeldin A were purchased from Sigma. Nocodazole and cytochalasin D were purchased from Calbiochem (San Diego, CA). EndoH was from New England Biolabs (Beverly, MA).

Immunofluorescence microscopy

For observation using confocal microscopy, cells were plated and cultured in two-well Lab-Tek chamber slides (Nalge Nunc Intl, Naperville, IL) and were then stained by double indirect immunofluorescence methods, as described previously (Valencia *et al.*, 2006). After washing in phosphate-buffered saline, cells were fixed in 4% paraformaldehyde at 4°C. Cells were then permeabilized with 0.01% Triton-X 100 for 3 minutes at 23°C and were blocked with a solution containing 5% normal goat serum and 5% normal horse serum for 1 hour at 23°C. Cells were incubated with primary antibodies (at dilutions noted in the figure legends) overnight at 4°C. Goat anti-rabbit IgG labeled with Texas red and horse anti-mouse IgG labeled with fluorescein (1:100; Vector, Burlingame, CA) were used to detect polyclonal and mAbs, respectively. Nuclear counterstaining was performed with 4,6-diamidino-2-phenylindole (Vector). Reactivity was classified into three categories, according to whether they showed green, red, or yellow fluorescence; the latter was indicative of colocalization of the red and green fluorescence signals. All preparations were examined with a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany), equipped with HeNe, argon, krypton, and UV laser sources. To visually observe the acidity of intracellular compartments, cells were incubated for 20 minutes at 37°C in MEM with 5 μg/ml acridine orange (Fuller *et al.*, 2001). After extensive washing with phosphate-buffered saline, coverslips were mounted onto slides. Fluorescence was observed using a Leica DMR B/D MLD fluorescence microscope (Leica Microsystems, Bannockburn, IL).

Western blotting

Cell extracts were prepared for SDS-PAGE analysis as described previously (Watabe *et al.*, 2004). Briefly, cell extracts were mixed in Tris-glycine SDS sample buffer (2 ×) (Invitrogen, Carlsbad, CA) supplemented with 2-mercaptoethanol and boiled for 5 minutes. Samples (10 μg protein/well) were loaded into 8% or 8–16% gradient SDS-PAGE gels (Invitrogen), and were then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were placed in blocking solution (5% non-fat dry milk in 10 mM Tris-HCl, pH 7.2; 150 mM NaCl; 1% Tween 20) for 1 hour at 23°C and were then incubated with primary antibodies (diluted as noted in the figure legends) overnight at 4°C. Membranes were incubated in horseradish peroxidase-linked anti-rabbit, anti-mouse whole antibodies (1:1000) (Amersham, Piscataway, NJ), or anti-goat immunoglobulin (DAKO, Carpinteria, CA), for 1 hour at 23°C. Bound antibodies were detected using an ECL-plus Detection System (Amersham), according to the manufacturer's instructions.

Electron microscopy and DOPA staining

This procedure was performed as reported previously (Kushimoto *et al.*, 2001). Briefly, cells were harvested in phosphate-buffered saline and fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Samples were then stained with 0.1% L-DOPA (Sigma) for 2 hours and were washed with 0.1 M cacodylate buffer. Samples were then embedded in epoxy resin. Thin sections were cut, stained with uranyl acetate, and then examined with a Zeiss EM 912 EX electron microscope.

Glycosidase digestion

Cell extracts (2 μg protein) were digested with 1000 U EndoH for 3 hours at 37°C. After digestion, extracts were subjected to SDS-PAGE as described above.

Metabolic labeling and immunoprecipitation

Metabolic labeling and immunoprecipitation were performed as described previously (Costin *et al.*, 2003). Briefly, cells were incubated in Met/Cys-free DMEM medium containing 10% dialyzed FBS (GIBCO) for 30 minutes at 37°C and were then labeled for 30 minutes at 37°C with 0.5 mCi of [³⁵S]Met/Cys (Amersham). Cells were chased at various times at 37°C (as detailed in the figure legends), in medium supplemented with 1 mM methionine. At this time, cells were treated, where indicated, with 10 μg/ml brefeldin A, 10 μg/ml nocodazole, or 10 mg/ml cytochalasin D. Cells were harvested and solubilized overnight at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.01% SDS, containing complete protease inhibitor cocktail). Cell extracts were cleared for 2 hours at 4°C first with normal rabbit serum (Vector) and then with Protein-G Sepharose beads (Amersham). Proteins were immunoprecipitated with either αPEP7h or αPEP13h and complexes were separated with Protein-G Sepharose beads for 2 hours at 4°C. Pellets were prepared for SDS-PAGE analysis as described above.

Subcellular fractionation

Isolation of melanosomes, clathrin-coated vesicles, and endosomes were performed as described previously, without modifications (Watabe *et al.*, 2004). Purification of plasma membranes by the sucrose and iodixonadol gradients was performed as described previously, also without modifications (Valencia *et al.*, 2006).

Microarray hybridization and data analysis

The detailed procedure for DNA microarray analysis used is available at <http://nciarray.nci.nih.gov/reference/NCIReference.shtml>. Briefly, 10 µg total RNA, isolated using an Rneasy Mini Kit (Qiagen, Valencia, CA), from αMSH- or ASP-treated, or from untreated melan-a melanocytes were used to derive fluorescently (Cy5 or Cy3) labeled cDNAs, which were hybridized on Mouse Exonic Evidence-Based Oligonucleotide (MEEBO) arrays. These arrays contain a genome-wide set (>38 K) of long-mer mouse oligonucleotides and were manufactured and printed at the Advanced Technology Center (National Cancer Institute, Gaithersburg, MD). At least two hybridizations were performed for each of the three biological replicates using a dye-swap strategy to eliminate labeling bias of the fluorescent intensity measurement. Hybridized arrays were scanned at 10 µm resolution on a GenePix 4000B microarray scanner running GenePix 5.0 software (Axon instruments, Union City, CA) at variable photomultiplier tube voltage to obtain the maximal signal intensities with 1% probe saturation. Another scan was performed at a lower photomultiplier tube setting (pmt) to avoid any saturated spot, in order to get the complete set of altered genes. For each spot, the highest value of each non-saturated spot was used. Array data (images and sample intensity files) were entered into the NCI microarray database (mAdb, <http://nciarray.nci.nih.gov/index.shtml>), to be filtered and normalized. Our quality filter required each spot to have a signal to background ratio of at least 2 for one of the two channels and at least 50 foreground pixels. Cy3: Cy5 intensity ratios were normalized by median centering of ratios for each array. After inverting the ratios of the reciprocal experiment, the data were transformed to logarithmic scale to the base 2 for statistical calculations. Differentially expressed genes between αMSH- or ASP-treated versus control samples were identified using one-sample *t*-tests. Treated-to-control sample ratios were calculated using geometric averages of the replicates. Changes in gene expression are considered significant (at $P \leq 0.01$) when up- or downregulated by at least 1.5-fold by ASP and/or by αMSH, after averaging the replicates, and data were present at least for half of the six replicates. Global expression changes are considered to be statistically significant, since the number of genes differentially expressed at $P < 0.01$ were found to be about five-fold larger than estimated false discoveries at this *P*-value.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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