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Sorting of Pmel17 to melanosomes through the plasma membrane by AP1 and AP2: evidence for the polarized nature of melanocytes

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Summary

Adaptor proteins (AP) play important roles in the sorting of proteins from the trans-Golgi network, but how they function in the sorting of various melanosome-specific proteins such as Pmel17, an essential structural component of melanosomes, in melanocytes is unknown. We characterized the processing and trafficking of Pmel17 via adaptor protein complexes within melanocytic cells. Proteomics analysis detected Pmel17, AP1 and AP2, but not AP3 or AP4 in early melanosomes. Real-time PCR, immunolabeling and tissue in-situ hybridization confirmed the coexpression of AP1 isoforms μ1A and μ1B (expressed only in polarized cells) in melanocytes and keratinocytes, but expression of μ1B is missing in some melanoma cell lines. Transfection with AP1 isoforms (μ1A or μ1B) showed two distinct distribution patterns that involved Pmel17, and only μ1B was able to restore the sorting of Pmel17 to the plasma membrane in cells lacking μ1B expression. Finally, we established that expression of μ1B is regulated physiologically in melanocytes by UV radiation or DKK1. These results show that Pmel17 is sorted to melanosomes by various intracellular routes, directly or indirectly through the plasma membrane, and the presence of basolateral elements in melanocytes suggests their polarized nature.

Keywords

Pmel17; Plasma membrane; AP1; AP2; Melanocytes

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Introduction

Epithelial cells constitute a protective barrier against the environment, but also serve as exchange interfaces with the outside world. To fulfill these functions, epithelial cells have evolved characteristic apical and basolateral plasma membrane regions. The basolateral membrane contacts neighboring cells and underlying tissue, whereas the apical membrane generally faces the lumen of an internal organ (Schuck and Simons, 2004). Melanocytes are epithelial cells that provide a first defense against solar exposure by distributing their pigment to overlying keratinocytes, and by communicating with other epithelial cells in the epidermis (Hearing, 2000). Melanocytes possess two functionally distinct types of cell surface: dendrites that are involved in the transfer of melanosomes to neighboring keratinocytes, and a central perinuclear region that is probably involved in cell-to-cell recognition. Owing to the similarities between polarized epithelial cells and melanocytes, we evaluated the expression of polarizing sorting elements in melanocytic cells and their relation to the sorting of melanosomal proteins.

Pmel17 (also known as gp100) is a type I integral membrane protein that is part of the internal matrix observed in stage II melanosomes (Kushimoto et al., 2001; Berson et al., 2001). Pmel17 trafficking is intriguing because it accumulates both in multivesicular bodies (MVB) and in early endosomes (Raposo et al., 2001), organelles involved in protein sorting to the endocytic and secretory pathways, respectively. Furthermore, Pmel17 is the only known melanosomal component that exists in two forms [membrane-bound ~100 kDa mature Pmel17 (mPmel17) and secreted or soluble ~76 kDa (sPmel17)] that differ in their N-glycan composition (Maresh et al., 1994). These considerations suggest that Pmel17 sorts through both the secretory and endocytic pathways with relatively high efficiency; however, the sorting mechanisms and proteins involved in this differentiated trafficking are not known.

Membrane protein sorting at the trans-Golgi network (TGN) is controlled by cytoplasmic domain-targeting signals, which contain crucial tyrosine and/or di-leucine residues that are often decoded by cytosolic heterotetrameric complexes of adaptor proteins (AP), which consist of two large (α, γ, δ or ε, and β), 1 medium (μ) and 1 small (σ) subunit (Doray and Kornfeld, 2001; Bonifacino and Traub, 2003). Tyrosine-based signals are mainly recognized by the μ subunits of adaptor proteins (Sugimoto et al., 2002), whereas di-leucine-type signals [DE]xxxL[LI] can interact with the μ subunits of AP1 or AP2, with a combination of the γ and σ1 subunits of AP1 or with the δ and σ3 subunits of AP3 (Janvier et al., 2003). These signals mediate endocytosis and are implicated in basolateral targeting in polarized epithelial cells. Several melanosome-specific proteins, such as tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and Pmel17 have one or both of those sorting signals in their sequences (for a review, see Bonifacino and Traub, 2003).

Four adaptor protein complexes (AP1, AP2, AP3 and AP4) have been identified thus far (Dell'Angelica et al., 1999). AP1, AP3 and AP4 act at the level of the TGN or endosomes, whereas AP2 acts at the plasma membrane (Brodsky et al., 2001). The recent discovery of the μ1B subunit of AP1, which can assemble with the three common subunits of AP1A (γ, β1 and σ1) to generate the AP1B complex, is relevant to the role of AP1. The two forms of

AP1 (AP1A and AP1B) have quite distinct functions: AP1A is ubiquitously expressed, whereas AP1B is an epithelial cell-specific complex involved in polarized sorting to the plasma membrane (Fölsch et al., 2003).

The role of adaptor proteins in melanosomal protein transport was first described in melanocytes derived from patients with Hermansky-Pudlak Syndrome type 2 (HPS-2) who carry mutations in the β3A subunit of AP3. In those cells, TYR is misrouted to multivesicular bodies (Huizing et al., 2001). However, HPS-2 melanocytes do not show alterations in Pmel17 distribution, suggesting that sorting of Pmel17 is distinct from TYR, perhaps using another adaptor protein complex. In that regard, Pmel17 has been proposed to sort directly to stage I melanosomes (Kushimoto et al., 2001) and/or indirectly via late endosomes (Berson et al., 2001). However, neither of those trafficking routes explain the presence of Pmel17 fragments at the cell surface which are internalized by receptor mediated endocytosis in melanoma cells (Ramakrishna et al., 2004).

We now demonstrate the coexpression of both AP1A and AP1B in melanocytic cells and their active involvement in the sorting of Pmel17, which supports the polarized nature of melanocytic cells. Thus, Pmel17 uses several distinct sorting pathways to reach early melanosomes via clathrin coated vesicles (CCV), one involving rapid and direct sorting of Pmel17 to melanosomes (via AP1A), whereas the other involves an indirect sorting of the Pmel17 to the plasma membrane, (via AP1B) and then redirection to melanosomes (via AP2). Furthermore, we confirm that μ1B expression is regulated by physiological factors that control the expression of Pmel17 in epidermal melanocytes.

Results

AP1 and AP2 are involved in the transport of Pmel17

Melanosomes mature from undifferentiated and non-pigmented organelles (called Stage I melanosomes) to differentiated and highly pigmented organelles (Stage IV melanosomes). These organelles are divided into ‘early’ (i.e. Stage I or II) or ‘late’ (i.e. Stage III or IV) melanosomes which lack or contain melanin pigment, respectively. The final intracellular destination of Pmel17 is the stage I melanosome, which has a short half-life before it matures further in pigmented melanocytic cells, such as melanocytes and MNT1 melanoma cells. However, unpigmented melanocytic cells, such as mutant melanocytes and SkMel-28 melanoma cells, accumulate large quantities of early stage melanosomes owing to their dysfunctional maturation.

Using early and late melanosomes from those cell lines, we used mass spectrometry to identify proteins involved in trafficking and sorting to early melanosomes and compared those with components of late melanosomes. Proteomics analysis of these melanosomes revealed more than 600 proteins and those relevant to sorting are summarized in supplementary material Tables S1 and S2. Adaptor protein family proteins detected in stage I melanosomes included AP1 and AP2. Interestingly, the presence of proteins sorted through the secretory pathway, such as the low-density lipoprotein receptor related protein 1 (LRP), the transferrin receptor (TfR) and its homolog melanotransferrin, in stage I melanosomes,

indicates the existence of a unique pathway in melanocytes and suggests complex transport/sorting mechanisms for melanosomal proteins.

To validate these proteomics results, we confirmed the presence of Pmel17, AP1, AP2 and TfR in all stages of melanosomes and in CCV isolated from M14 cells and MNT-1 cells (Fig. 1A,B). Next, we examined whether Pmel17 could form complexes with AP1 and/or AP2. Sequential immunoprecipitation analysis, under denatured and reduced conditions, revealed that Pmel17 formed complexes with clathrin, the γ subunit of AP1 (γ-adaptin), and the α subunit of AP2 (α-adaptin) (Fig. 1C). These results are consistent with evidence that di-leucine motifs, as found in Pmel17, preferentially bind the γ and σ subunits rather than the μ1 subunit of AP1 (Janvier et al., 2003). Double immunofluorescence showed that Pmel17 colocalized with γ-adaptin and α-adaptin, mainly in the perinuclear area and in parts of the cytoplasm (yellow in Fig. 1D), but much less in the dendrites of M14 cells and of MNT-1 cells. The α-adaptin antibody showed a marked localization in the plasma membrane and cytoplasm. Note, that neither AP1 nor AP2 showed complete colocalization with Pmel17.

We performed immunoelectron microscopy to confirm the localization of AP1 and AP2 and its relationship with early melanosomes in melanoma cells (Fig. 2). Pmel17 and AP1 were found in the membrane of CCV (Fig. 2A) and stage I melanosomes (Fig. 2B). The presence of AP1-positive vesicles inside stage I melanosomes suggests that AP1 may also be involved in the exchange/retrieval of proteins from these organelles (Fig. 2B, inset). Interestingly, the amount of gold-labeled AP1 at the membrane of stage II melanosomes (Fig. 2C) was less than that observed in stage I melanosomes (Fig. 2C, inset).

We found coated vesicles containing both Pmel17 and AP2 in the cytoplasm (Fig. 2D) and on the outer membranes of stage I melanosomes (Fig. 2E, inset), which suggests that Pmel17 is a cargo protein of AP2 and that it arrives directly from the plasma membrane to these organelles. Interestingly, we observed AP2 gold particles at stage II melanosomes (Fig. 2F), that were in some cases clustered within the organelle (Fig. 2F, inset arrows).

Taken together, these results indicate that Pmel17 is transported to melanosomes in CCV that contain either AP1 or AP2, which form complexes with Pmel17, and that one or both of those adaptor proteins are involved in Pmel17 trafficking to early melanosomes.

Dual transport of Pmel17 is mediated by AP1

The presence of AP1 in early melanosomes and the interactions of Pmel17 with AP1, as shown above, prompted us to analyze the expression of μ1 subunit isoforms (μ1A and μ1B) in several types of melanocytic cell, in NHEK and in retinal pigment epithelium (RPE) cells.

Since specific antibodies to μ1A are not available, we used real-time PCR to determine whether the μ1A subunit is expressed in those cell lines (Fig. 3A). Interestingly, 1106c human primary melanocytes showed higher levels of μ1A subunit RNA expression compared with melanoma cells and NHEK. To analyze its subcellular distribution, we transiently transfected M14 cells and MNT-1 cells with cDNA of μ1A tagged with HA (Fölsch et al., 1999). Immunoblot analysis with an anti-HA antibody detected a ~50 kDa

band only in transfected cells (Fig. 3B). Dual immunofluorescence analysis revealed that μ1A (green) was mainly located in the perinuclear area, where it colocalized strongly with Pmel17 (red) (Fig. 3D,H) in both cell lines but little colocalization was seen in the peripheral areas of the cells (Fig. 3E,I). This pattern is consistent with the localization of AP1A in the TGN and to a lesser extent in endosomes (Fölsch et al., 2001).

By contrast, μ1B RNA was detected only in four out of six cell lines tested, including 1106c and NHEK (Fig. 4A). In those four cell lines, the RNA levels of μ1B were 100 times lower than those for μ1A. RPE cells, which are known to have a polarized organization, are negative for μ1B RNA. To assess the presence of μ1B at the protein level, we used metabolic labeling and immunoprecipitation (Fig. 4B), which detected a strong band of ~50 kDa (arrowhead) in lysates of μ1B-positive cells but no such band in the μ1B-negative cells. Confocal microscopy analysis showed that μ1B (green) was distributed mainly in the perinuclear region (Fig. 4D,G), outside the central Golgi area where μ1A was localized, with a less pronounced presence in dendrites. This pattern indicates that μ1B is located in a post-Golgi compartment, which is consistent with its location in recycling endosomes (Fölsch et al., 2003; Ang et al., 2004). Furthermore, we found vesicles that were double positive for μ1B and Pmel17 near the plasma membrane, but not in dendrites, in these cells (Fig. 4E,H, arrows).

Taken together, the coexpression of both AP1A and AP1B isoforms in melanocytic cells, especially in primary melanocytes and in NHEK indicates their polarized nature. Furthermore, the different localization patterns of AP1A and AP1B suggest that Pmel17 transport by AP1 complexes may be through two distinct routes.

AP1B rescues the sorting of low-density lipoprotein receptor (LDLR) and Pmel17 in SKMel-28 cells

AP1B interacts with LDLR for basolateral sorting to the plasma membrane in polarized cells, although it is sorted to the apical plasma membrane in non-polarized cells (Fölsch et al., 1999). This prompted us to characterize the role of AP1B regarding the distribution of LDLR and Pmel17 in μ1B-positive (M14) and in μ1B-negative (MNT-1 and SKMel-28) melanoma cells. Confocal microscopy showed that LDLR and Pmel17 were diffusely distributed throughout the cytoplasm and the plasma membrane in M14 cells and in MNT-1 melanoma cells (Fig. 5A), but were mostly in the perinuclear area of μ1B-negative SKMel-28 cells. Cross-section analysis revealed that LDLR and Pmel17 were sorted to the upper plasma membrane, where LDLR colocalized with Pmel17 (Fig. 5B, arrows).

We then investigated whether re-expression of μ1A or μ1B in the μ1B-negative SKMel-28 cells would rescue the distribution observed for LDLR and Pmel17 in μ1B-positive M14 cells. We transiently transfected μ1A or μ1B in SKMel-28 cells and analyzed the subsequent intracellular distribution of LDLR and Pmel17 by dual IF (Fig. 5C). Transfection of μ1A did not affect the localization of LDLR. By contrast, transfection of μ1B produced a marked redistribution of both LDLR and Pmel17 throughout the cytoplasm and at the plasma membrane (arrows), but especially in the dendrites, that resembled the pattern observed in μ1B-positive M14 cells. Thus, transfection of μ1B, but not μ1A, rescues the cytoplasmic localization of both LDLR and Pmel17 in SKMel-28 cells.

These results demonstrate that Pmel17 is a cargo protein of AP1B in melanoma cells. However, the existence of other sorting determinants in Pmel17, such as glycosylation (Gut et al., 1998), provides a way of sorting Pmel17 through the secretory pathway in the absence of AP1B expression, such as observed in MNT-1 cells.

The partially glycosylated form of Pmel17 is transported to the plasma membrane

Pmel17 is a cargo protein of AP1B and AP2, and because these adaptor proteins are associated with transport to the plasma membrane, we decided to confirm whether Pmel17 is present in the plasma membrane from cells expressing (M14 cells) or not expressing (MNT-1 cells) μ1B. We isolated plasma membrane proteins using two methods, one based on a cross-linking reaction and another based on density gradients. Pmel17 was recovered from the cell surface using a biotinylated reagent and represented ~45% of the total amount of Pmel17 in those two cell lines (Fig. 6A) as estimated from the amount of integrin 5α (I5α) present in the plasma membrane fraction relative to the total amount in the cells. Interestingly, the majority of Pmel17 recovered was not from the mPmel17, but from a lower molecular weight form (solid black arrow) from M14 cells and from MNT1 cells, which is similar to the 76 kDa sPmel17 form reported earlier (Maresh et al., 1994).

Similarly, the partially glycosylated form of Pmel17 was again detected using a combination of sucrose plus iodixonadol density gradients (SIG) from both cell lines (Fig. 6B). In this case, the purity of the plasma membrane fraction was assessed using plasma membrane resident proteins, I5α and VLA2α (Fig. 6C). The SIG fraction is also enriched in clathrin, α-adaptin (AP2), and γ-adaptin (AP1). Interestingly, the presence of TfR and LDLR, known to sort to the plasma membrane in polarized and in non-polarized cells (Strickland et al., 2002), shows that SIG is an effective method for isolation of plasma membrane proteins.

Together, these results show that Pmel17 is sorted to the plasma membrane in melanoma cells. In addition, the isolation of only the partially glycosylated form of Pmel17 strongly suggests that glycosylation, in the absence of functional μ1B, may play a role in determining the sorting of Pmel17 to the plasma membrane as reported previously (Gut et al., 1998).

UV radiation upregulates the expression of μ1B in epidermal melanocytes and NHEK in human skin

The expression of μ1B RNA both in melanocytes and in keratinocytes, and the critical cell-to-cell communications between these cells under normal conditions and following exposure to UV radiation (Hearing, 2000), led us to analyze the effects of UV on μ1B expression. We examined skin biopsies from patients with or without UV irradiation using tissue in-situ hybridization (TISH). In normal skin, keratinocytes are located in the epidermal layer, whereas melanocytes are located at the dermal:epidermal border and are readily detected using a Pmel17 riboprobe (Fig. 7A). In non-UV-irradiated skin, melanocytes and keratinocytes showed a specific cytoplasmic signal for μ1B in their cytoplasm (Fig. 7B). Interestingly, there was an increased expression of μ1B both in melanocytes and in keratinocytes in skin irradiated with UV (Fig. 7C). In the dermis, μ1B was expressed in sebaceous glands (Fig. 7E, arrows), which serve as positive controls in those sections, and in polarized epithelial cells of human kidney (Fig. 7F).

Together these results confirm that the expression of μ1B in melanocytes and in keratinocytes is upregulated physiologically by UV in the epidermis, and support an important role for μ1B in the response of the skin to UV radiation.

DKK1 modulates the expression of adaptor proteins and μ1B in human melanocytes

Dermal fibroblasts regulate melanocyte function and proliferation through the secretion of DKK1, an inhibitor of Wnt signaling (Yamaguchi et al., 2004). We analyzed differences in expression of the adaptor protein gene after treatment of primary melanocytes with DKK1 using cDNA microarray analyses. Those analyses confirmed the expression of genes encoding all subunits of the four known adaptor protein complexes, including the μ1B subunit (Table 1). The AP4 β1 subunit gene was upregulated most in response to DKK1, with an increase of 2.9-fold, followed by AP2 α1 (1.75-fold), AP3β2 (1.73-fold) and two AP1 subunits γ1 and μ1B (1.51- and 1.44-fold, respectively). Interestingly, those three adaptor proteins are mainly involved in protein sorting through the secretory pathway. The upregulation of LDLR family members, such as LRP1B (2.15-fold), LRP12 (2.13-fold) and LDLR (1.62-fold), which are involved in cell surface endocytosis and regulation of Wnt signaling (Zilberberg et al., 2004), was also seen following treatment with DKK1. By contrast, μ1A expression was downregulated (0.78-fold) by DKK1 and three subunits of AP3 (μ2, β1 and δ1) were among the most downregulated adaptor protein family members, which are involved in trafficking through the endocytic pathway.

Thus, the expression of adaptor protein family members, including μ1A and μ1B, is physiologically regulated in melanocytes by DKK1 and responses of melanocytic cells to DKK1 may in part be based on regulation of protein trafficking through the secretory pathway.

Discussion

Melanosome proteomics identified APs associated with Pmel17 sorting

Melanosome purification has been challenging because of the lack of specific markers that recognize specific stages of melanosome maturation. Furthermore, the hybrid nature of melanosomes as lysosome-related organelles (Dell'Angelica et al., 2000; Barral and Seabra, 2004), and the involvement of multivesicular bodies in melanosome biogenesis (Berson et al., 2001), may explain the presence of early and late endosome markers in purified melanosomes. Furthermore, the identification of intraluminal vesicles in early melanosomes (Raposo et al., 2001) may well be the source of proteins originating from both the endocytic and secretory pathways found in melanosomes. To resolve this problem, we analyzed proteins present in early melanosomes and compared those with proteins in late melanosomes. This approach allowed us to obtain a list of melanosome-related proteins from which we analyzed the presence of adaptor protein family members. Immunoelectron microscopy confirmed that AP1 and AP2 are present in early melanosomes and derive from transport vesicles carrying Pmel17 to those organelles.

Pmel17 uses two distinct adaptor proteins to reach melanosomes

The fact that Pmel17 complexes with AP1 and/or AP2 to reach stage I melanosomes suggests for the first time that this melanosomal protein follows different sorting pathways. Pmel17 is probably not unique in this regard because lysosomal membrane proteins also follow different routes to reach their targets (Hunziker and Geuze, 1996). The co-expression of AP1A and AP1B in primary melanocytes and in some melanoma cells may be the key to understanding this differentiation process. AP1A and AP1B are functionally distinct, even though they differ only in the μ subunit, and exhibit great specificity for endosomal transport versus cell polarity (Fölsch et al., 2003). The distinctive patterns of AP1A and AP1B in transfected melanoma cells and its interaction with Pmel17 led us to propose that the rapidly processed mPmel17 is sorted directly to stage I melanosomes via clathrin-coated vesicles (probably containing μ1A). By contrast, sPmel17 is transported either to the plasma membrane or to recycling endosomes via AP1 complexes containing μ1B and is then endocytosed in clathrin-coated vesicles containing AP2. Thus, the endocytosed Pmel17 might first reach early endosomes (Raposo et al., 2001) or might be sorted directly to stage I melanosomes in a fashion similar to lysosomal acid phosphatase that uses only the secretory pathway to reach lysosomes from the plasma membrane (Hunziker and Geuze, 1996). Since AP2 is only associated with the plasma membrane, the presence of AP2 vesicles in stage I melanosomes supports the direct trafficking of Pmel17 to the endosomal-lysosomal system throughout the cell surface, a pathway recently validated for MHC class II molecules (McCormick et al., 2005).

The existence of this novel sorting mechanism for Pmel17 is clearly not a misrouting of the protein to the plasma membrane. Another melanosomal protein, TYRP1 is present in the plasma membrane of melanocytic cells (Vijayasaradhi et al., 1995), but the mechanism for this localization are not known. Further, melanocytes derived from HPS-2 patients showed severely altered trafficking in the endosomal pathway, which increased trafficking of lysosomal proteins (e.g. LAMPs 1–3) to the plasma membrane, but only affected TYR, not Pmel17 (Huizing et al., 2001). A second, but less likely, hypothesis is that increased production of Pmel17 might saturate the primary direct route to melanosomes, and may redirect the excess protein to the plasma membrane for secretion (Maresh et al., 1994).

Multiple sorting determinants are responsible for Pmel17 sorting to the plasma membrane in melanoma cells

The presence of Pmel17 in the plasma membrane of μ1B-negative cells indicates that AP1B is not the only sorting determinant for Pmel17. In these cells, the loss of μ1B expression may trigger a generalized sorting of Pmel17 to the entire surface of the plasma membrane, instead of a more defined localization in μ1B-expressing melanocytic cells. As an example of the importance of correct sorting to the plasma membrane, missorting of LDLR in hepatic cells of patients with familial hypercholesterolemia creates severe consequences (Strickland et al., 2002). Sorting of Pmel17 to the plasma membrane may rely on the existence of other sorting determinants encoded in protein transmembrane domains, attached lipid moieties, or carbohydrate domains (Gut et al., 1998). Evidence for these sorting determinants in Pmel17 was initially supported by the identification of sPmel17, which contains a different glycan composition compared with the cytoplasmic mPmel17 (Maresh et al., 1994) and is

consistent with our identification of a partially glycosylated form of Pmel17 in the plasma membrane of melanoma cells. Transfection of μ1B restored the correct plasma membrane distribution for LDLR and Pmel17 in the central area of SKMel-28 cells.

Implications of physiologic regulation of μ1B expression in melanocytes

Our data show that the low levels of μ1B RNA found in vitro and the difficulty to detect it under normal culture conditions is due to its physiological regulation by exogenous stimulants or inhibitors of melanocyte function, such as UV radiation and DKK1, respectively. This suggests that μ1B may be responsive to external stimuli and may modulate the lateral sorting pathway to regulate the expression of proteins in the plasma membrane.

Thus, the trafficking and secretion of melanosomes during maturation from the perinuclear area to dendrites in melanocytes, and the polarized location of melanosomes in keratinocytes after UV exposure (known as melanin ‘capping’) are both better explained by considering the polarized nature of those cells. In this regard, expression of μ1B may also have important implications with respect to melanoma progression. Cadherins and connexins are delivered to the plasma membrane via the basolateral sorting pathway (Bryant and Stow, 2004), and disruption of that pathway may have consequences in the cross-talk of melanocytes with keratinocytes in normal skin and in the regulation of melanocyte proliferation (Haass et al., 2004). The loss of such communication is closely related to malignant transformation and ultimately to metastasis. The fact that human primary melanocytes, NHEK, and early stages of melanoma cells express μ1B whereas cell lines derived from metastatic melanomas do not, has not escaped our attention. It will be important in the future to develop in vitro models for melanocytic cells that favor their polarized nature to further characterize the role of plasma membrane domains and to examine μ1B expression in normal and transformed melanocytes to see if its expression is correlated with aggressive and/or metastatic behavior.

In summary, our results provide new information about the complexity of Pmel17 sorting in melanocytic cells (schematically presented in Fig. 8). Pmel17 is sorted from the Golgi using at least two different sorting mechanisms to reach stage I melanosomes involving both the endocytic and secretory pathways. They further suggest that glycosylation may be an important element determining the sorting pathway followed, which is consistent with recent observations in protein trafficking in epithelial cells (Huet et al., 2003). Whether the partially glycosylated Pmel17 trafficked to the cell surface plays any role there in cellular function or immune recognition is also an interesting topic for future study. Further studies will be necessary to more fully elucidate the implications of these sorting mechanisms to organelle biogenesis and the malignant transformation of melanocytes.

Materials and Methods

Cell cultures

Pigmented (UACC 257 and MNT-1) and non-pigmented (M14 and SKMel-28) human malignant melanoma cells were cultured as described previously (Kushimoto et al., 2001; Watabe et al., 2004). Normal human epidermal keratinocytes (NHEK) were obtained from

MatTek (Ashland, MA). The pigmented HPM cell lines 1106c and 1108c were obtained from Zalfa Abdel-Malek (University of Cincinnati, Cincinnati, OH) and were cultured in 154 and 254 Medium supplemented with growth components (Cascade Biologicals, Portland, OR) for melanocytes and keratinocytes, respectively.

Human tissue samples and skin biopsies

Paraffin-embedded samples from human kidney and lung were obtained from the Tissue bank in Philadelphia, PA. Shave biopsies, 4 mm in diameter, were taken before and 1 day after a minimum erythema dose (MED) of UV, as described previously (Tadokoro et al., 2003).

Antibodies, reagents and primers

αPEP13h and αPEP7h antibodies recognize the C-termini of human Pmel17 and TYR, respectively (Kushimoto et al., 2001). Monoclonal antibodies used include HMB45 against Pmel17 (DAKO, Carpinteria, CA), T311 against TYR (Novocastra, Newcastle, UK) and LDLR (Oncogene, La Jolla, CA). Other monoclonal antibodies used are Bip/GRP78 (ER), Vti1b (Golgi), EEA1 (early endosome), Syntaxin 8 (late endosome), adaptin-α (AP1), adaptin-γ (AP2), VLA 2α and Integrin 5α (plasma membrane), all from Transduction Laboratories (Lexington, KY). The polyclonal antibody against the μ1B subunit of AP1 was a gift from Heike Fölsch (Yale University, New Haven, CT).

For TISH, the human AP1 μ1B (1275 bp fragment) and human Pmel17 (1986 bp fragment) probes were subcloned into the PCB6 and the pcDNA3.1 vectors (Invitrogen), respectively. The sequences for μ1B (T7) GGGCTTCTAGCTGGTACGAAGTTGG; (SP6) GTGTGGAGATATCTGTGCCTG, and Pmel17 (NM_006928) (T7) GGGCGGAACCTGCCCAAGGCCTGCT; (SP6) GGTGGAGACCACAGCTAGAGA were used as antisense (T7) and sense (S6) as noted. Riboprobes were synthesized as reported previously (Mutsuga et al., 2004). The PCR products for AP1 μ1B (349 bp) and Pmel17 (522 bp) were labeled using a digoxigenin RNA labeling kit, according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

For RT-PCR, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the supplier's instructions. Reverse transcription was performed with 400 ng cytoplasmic RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). After denaturation at 94°C for 4 minutes, PCR was performed for 36 cycles (30 seconds at 94°C, 30 seconds at 58°C, 40 seconds at 72°C) using Fast Start Taq polymerase (Roche, Mannheim, Germany). All amplified products were sequence verified. Determination of *AP1 μ* isoforms transcript levels was performed by semi-quantitative real-time RT-PCR using SyBr Green. The primers used were: mu1(GI# 18105005) forward, AAGGTGCTCATCTGCCGGAAC and reverse, TTGAAGTACTCGGAAAACACCTGCACC; mu1b (GI# 34783844) foward, AAGCCATTGATCAGCCGCAAC and reverse, TTGAAGTATTCGCAGAATACCTCTATTG; COOH mu1b forward, GTAGAGCTGGAGGATGTAAAATTC and reverse, CTTCTAGCTGGTACGAAGTTG; NH2 mu1b forward, ATGTCCGCCTCGGCTGTCTTCATT and reverse,

TGATTTGTTCTTGCTGCGGCCAGT. Quantified transcripts were relative to the human GAPDH gene using the following primers: forward, GAAGGTGAAGGTCGGAGTC and reverse, GAAGATGGTGATGGGATTTC; amplicon size, 226 bp (GI# 11564691) as reported previously (Rouzaud et al., 2003).

Microarray procedures

Modified oligo-DNA microarray analysis was performed as previously described (Yamaguchi et al., 2004). Total RNA quality (purity and integrity) was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technology, Waldbronn, Germany). Paired cDNA samples, labeled by cyanine 3-and cyanine 5-dUTP incorporation during reverse transcription, were hybridized simultaneously with one oligo-DNA chip (Hs-Operon V2-vB2.2p13) as per NCI protocol (available at http://mach1.nci.nih.gov/). Fluorescent scanning of the oligo-DNA chips was done using a microarray scanner (GenePix 4000A; Axon Instruments, Union City, CA). Differential gene expression was profiled with Genepix 3.0 software and analyzed by NCI-CIT software. Experiments were performed three times independently.

Tissue in-situ hybridization

TISH was carried out as described previously (Suzuki et al., 2002) with minor modifications. Briefly, cross sections of paraffin-embedded skins (3 μm) were deparaffinized, treated with proteinase K, and placed in 200 ml acetylation buffer (0.1 M triethylamine, pH 8.0, containing 0.25% acetic anhydride) for 15 minutes. After washing in 4× SSC for 10 minutes, samples were incubated in pre-hybridization solution (2× SSC, 50% deionized formamide) for 1 hour at 47°C. After overnight hybridization at 47°C, samples were placed in hybridization solution (Mutsuga et al., 2004) containing 10 μl purified DIG-labeled antisense riboprobe. Samples were then incubated in 10 mM Tris-HCl, 0.5 M NaCl and 0.25 mM EDTA (TNE) buffer, treated with RNaseA for 30 minutes, and returned to TNE buffer for 3 minutes, all at 37°C. After washing in 0.1 × SSC for 15 minutes at 47°C, samples were blocked for 30 minutes and incubated with anti-DIG/HRP conjugate (DAKO, Carpinteria, CA) for 40 minutes at room temperature (RT). For detection, we used the tyramide signal amplification system (GenePoint kit, DAKO) and VIP solution (Vector) according to the manufacturer's instructions. Samples were observed and photographed in a Leica DMRB microscope.

Cloning and transfection of μ1A and μ1B

PcB6 vectors containing the μ1A and μ1B subunits of AP1 (Fölsch et al., 2001) with an internal HA tag were obtained from Heike Fölsch (Yale University, New Haven, CT). Transient transfection of μ1A and μ1B into MNT-1 cells and into M14 cells was achieved by electroporation using the Nucleofector kits and equipment (AMAXA, Gaithersburg, MD) following the manufacturer's instructions.

Biochemical procedures

We isolated clathrin-coated vesicles and melanosomes as described previously (Watabe et al., 2004). The plasma membrane fraction resulted from a combination of sucrose gradient

centrifugation (Okada and Miyazaki, 1999) followed by iodixanol equilibrium gradient centrifugation as reported previously (Liang et al., 2003). Briefly, cells were harvested, homogenized, and centrifuged at 5000 ***g*** for 10 minutes at 4°C. The pellets were resuspended in 2.1 M sucrose, layered at the bottom of a 0.25 M, 1.25 M and 2.1 M gradient, and centrifuged at 75,000 ***g*** in a Beckman SW28 rotor for 16 hours at 4°C. Then, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and protein inhibitors (Sigma) were added to the 0.25 M sample and mixed with Optiprep™ (density 1.32 g/ml; ACSC, Westbury, NY). A self-generating gradient was formed in a 4 ml unsealed tube (Hitachi, Japan) at 500,000 ***g*** for 1 hour at 4°C. Layers were collected according to density markers (Amersham, Uppsala, Sweden).

For immunofluorescence, cells were seeded in two-well chamber slides (Nalgene, Naperville, IL) and were then fixed and stained as described previously (Valencia et al., 2001). Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C. After blocking with normal goat serum (NGS) or normal horse serum in PBS for 1 hour at RT, the cells were incubated with primary antibodies overnight at 4°C. Cells were then labeled with Texas Red anti-rabbit or FITC anti-mouse (Vector, Burlingame, CA) dyes for 1 hour at RT. Images were obtained using an LSM 510 confocal microscope (Zeiss, Jena, Germany). Colocalization signals were evaluated under equal microscope parameters using Zeiss colocalization software.

For immunoblotting (IB), cell extracts were prepared as previously published with modifications (Watabe et al., 2004). Briefly, samples were separated on 10% or 4–12% NuPAGE gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with casein for 1 hour at RT and were then incubated with primary antibodies overnight at 4°C. After washing with T-PBS (1 × PBS, 1% Tween 20, pH 7.2), blots were incubated in HRP-linked anti-rabbit or anti-mouse secondary antibodies (Amersham, Piscataway, NJ) for 1 hour at RT. Primary antibodies were detected using the ECL system (Amersham), according to the supplier’s instructions.

Post-embedding immunoelectron microscopy was performed as detailed previously (Tobin et al., 1996). Briefly, cells were harvested, fixed in 4% paraformaldehyde for 2 hours, dehydrated in cold ethanol, and embedded in LR white resin (Polysciences, Warrington, PA). Samples were cut and mounted in 200-mesh nickel grids, which were incubated with blocking solution (10% (w/v) NGS, and 0.1% BSA) for 30 minutes. Primary antibodies were incubated overnight at 4°C and then incubated with immunogold-conjugated secondary antibodies (1:25 dilution; Electron microscope Sciences, Fort Washington, PA) for 1 hour at RT. The grids were washed and stained in uranyl acetate and lead citrate. Images were taken with a Hitachi H7000 electron microscope (Tokyo, Japan) and equipped with a digital camera (Gatan, Pleasanton, CA).

Metabolic labeling with ^{35}S and immunoprecipitation (IP) were performed as previously reported (Watabe et al., 2004). plasma membrane proteins were recovered using a cross-linking reagent (Pierce) following the manufacturer’s protocol.

In-gel trypsin digestion and mass spectrometry

Early melanosomes (150 μg) were solubilized directly in sample loading buffer and were separated by 10% SDS-PAGE, according to the manufacturer's instructions (BioRad, Hercules, CA). Gels were stained with colloidal Coomassie Blue for 6 hours and were then destained in water. The lane containing the sample was cut into 15 slices from the top to the bottom of the gel. In-gel trypsin digestion was performed as previously described (Shevchenko et al., 1996). An aliquot of each digest was analyzed by a combination of a nano-HPLC/μESI ionization on a LCQ^{Deca} mass spectrometer (Thermo Electron Corporation, San Jose, CA) as previously described (Martin et al., 2000; Ficarro et al., 2002). The HPLC gradient (A=100 mM acetic acid in water, B=70% acetonitrile/100 mM acetic acid in water) was 0–5% B in 5 minutes, 5–40% B in 180 minutes, 40–60% B in 30 minutes, 60–100% B in 10 minutes, 100% B for 2 minutes, and 100-0% B in 5 minutes. Acquired data were searched against the human database (NCBI) with SEQUEST (Eng et al., 1994). The following parameters were used to evaluate results of the database search: DelMass<1.0, Xcorr>2.4, DelCn>0.1, Sp>500, RSp<10, Ion Ratio>0.6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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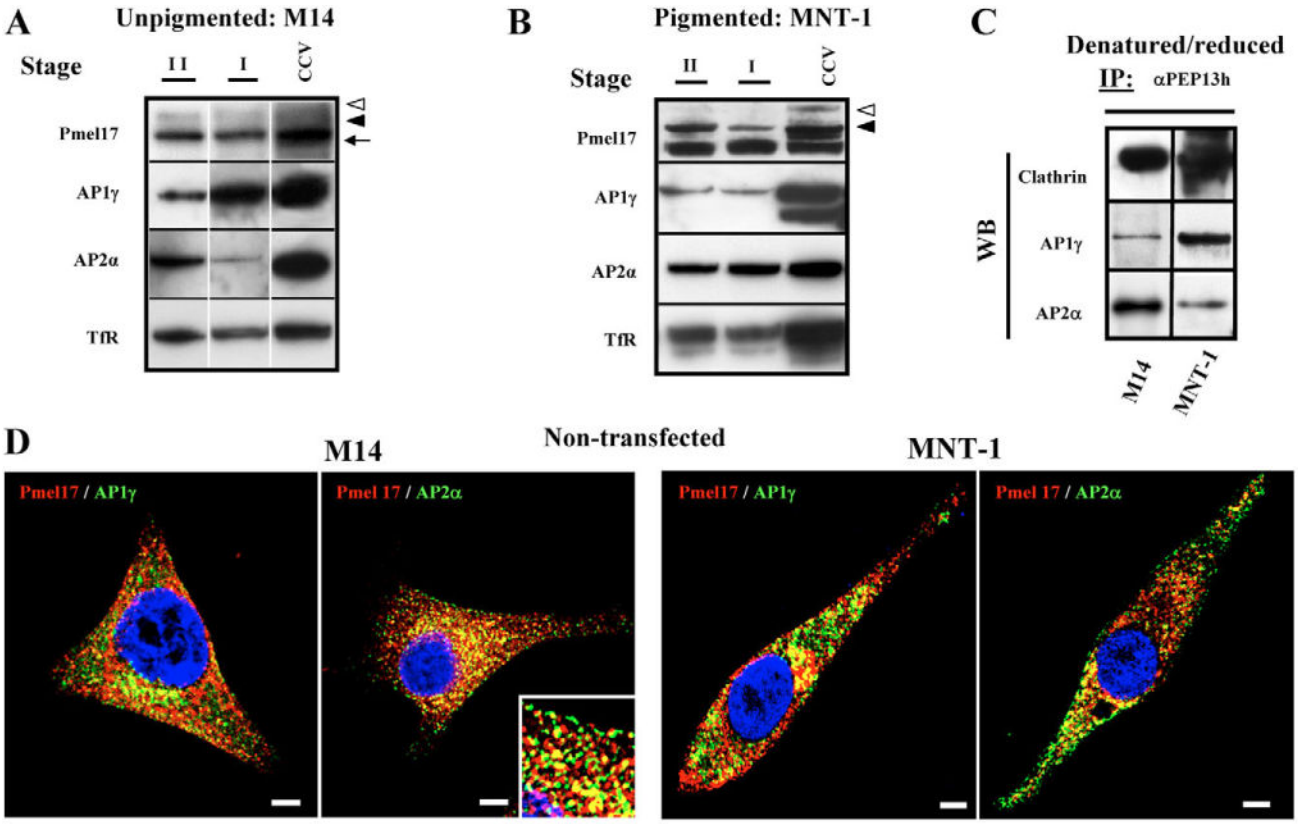

Fig. 1.

Protein components of melanosomes. (A,B) Early melanosomes and CCV isolated from M14 or MNT-1 cells were analyzed by IB with antibodies against Pmel17, AP1α, AP2α and TfR, as indicated. Experiments were performed twice with comparable results. (C) Pmel17 was immunoprecipitated using αPEP13h and was then immunoblotted with antibodies as noted. (D) Melanoma cells as noted were fixed and double stained for Pmel17 (red) and AP1γ or AP2α (green) as indicated. Representative images for each combination are shown. Bars, 10 μm.

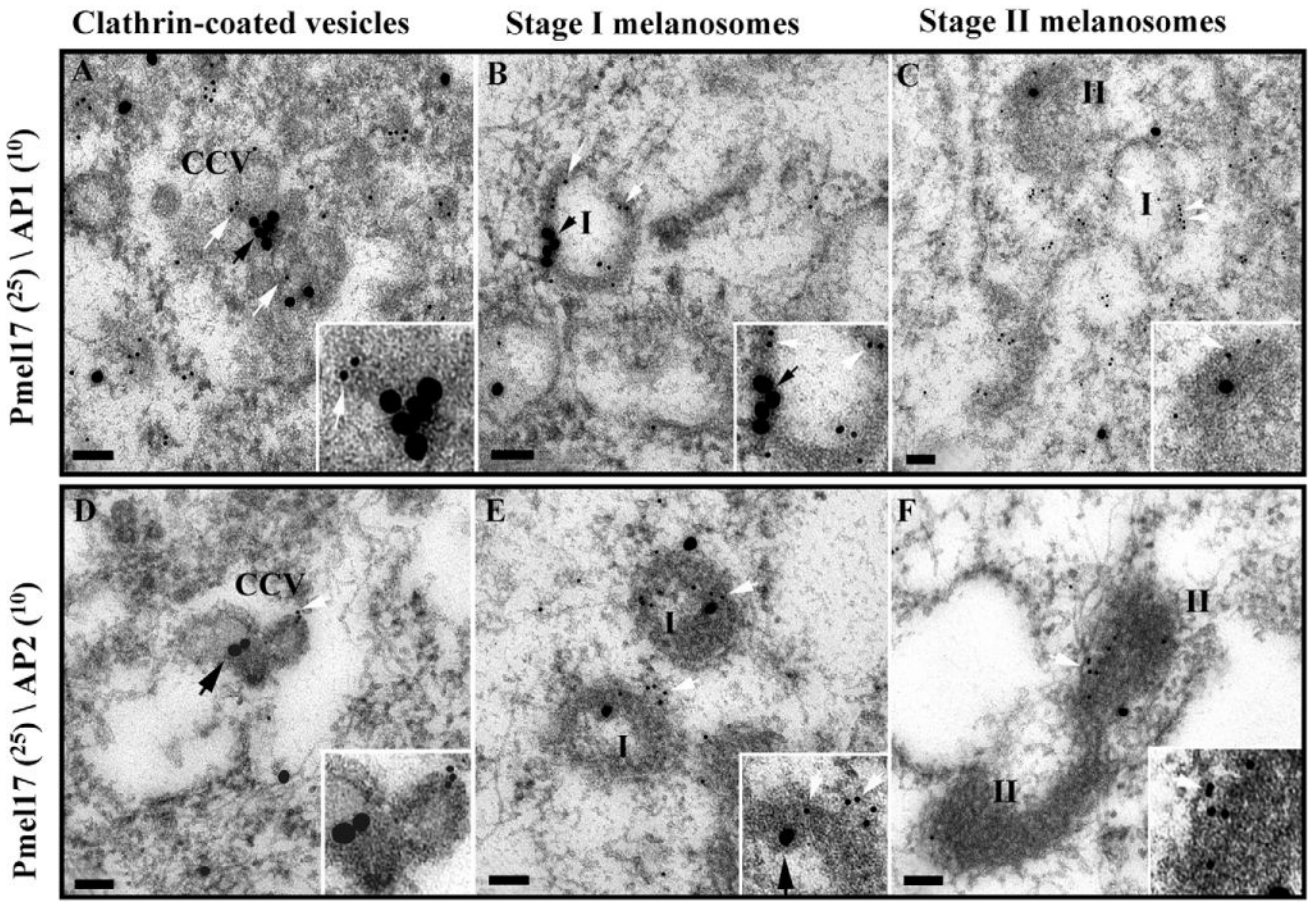

Fig. 2.

AP1 and AP2 colocalization with Pmel17. Melanoma cells were fixed, embedded in resin and double immunostained for Pmel17 with 25 nm gold (^{25}) and AP1 or AP2 with 10 nm gold (^{10}). Colocalization of Pmel17 (black arrows) with AP1 or AP2 (white arrows) was observed in CCV (A and D), stage I melanosomes (B and E) and stage II melanosomes (C and F). Note that AP2-vesicles with or without Pmel17 were observed in stage I melanosomes (E, inset) and inside vesicles in stage II melanosomes (F, inset). Bars, 0.1 μm.

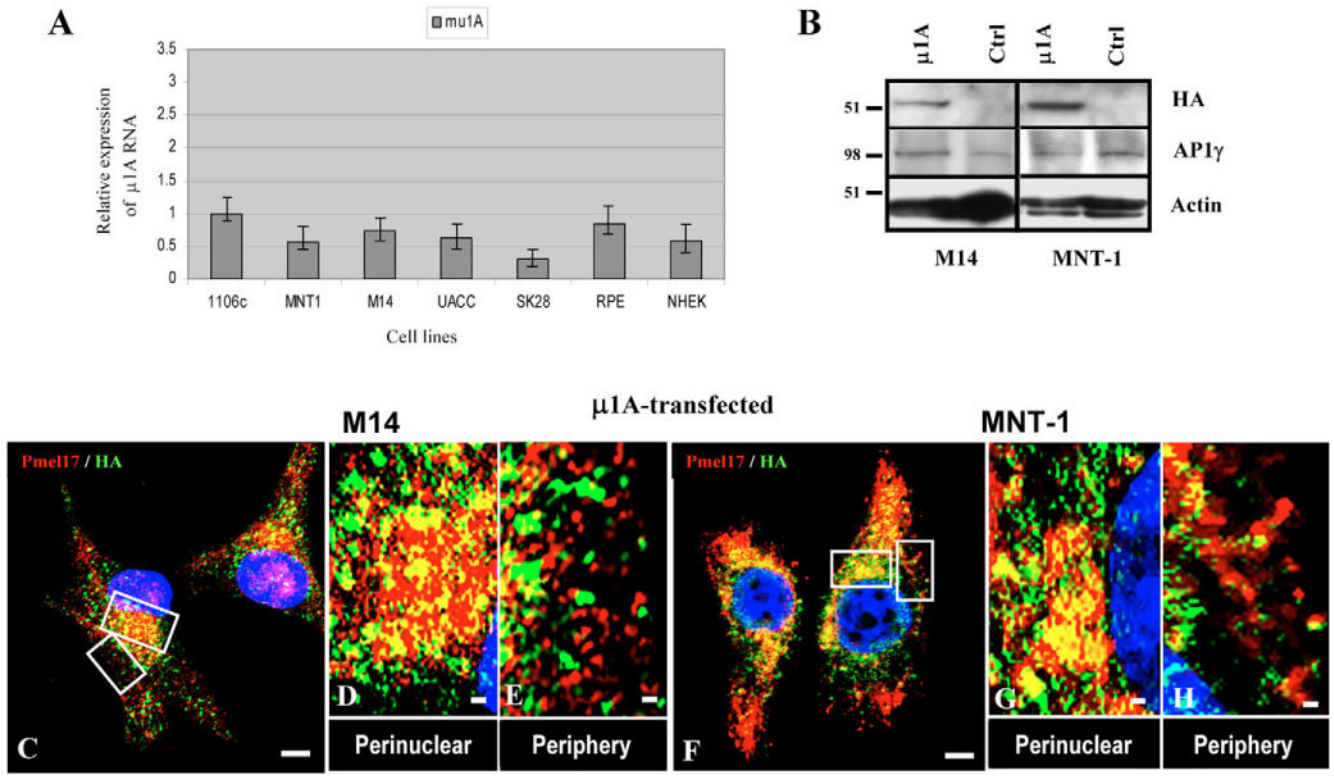

Fig. 3.

Expression of the μ1A subunit in melanocytic cells. (A) μ1A transcript levels determined by real-time PCR. Note the expression of μ1A in 1106c normal human melanocytes was higher than in other melanocytic cells or in NHEK. Values are mean ± s.d. of two experiments. (B) Melanoma cells as noted were transiently transfected with a plasmid containing μ1A tagged with HA, or without a plasmid as a control (Ctrl). Comparison of AP1γ and μ1A-HA subunits was performed by immunoblotting in those cell lines. Positions of molecular size markers are indicated on the left in kDa. (C–H) Melanoma cells as noted were fixed 48 hours after transfection with μ1A-HA, and stained with a combination of αPEP13h (red) and anti-HA (green) antibodies. Specimens were analyzed by confocal microscopy, and yellow fluorescence indicates colocalization. Nuclei were counterstained with DAPI (blue). Representative images are from at least two independent experiments. Bars, 10 μm (C,F); 1 μm (D,E,G,H).

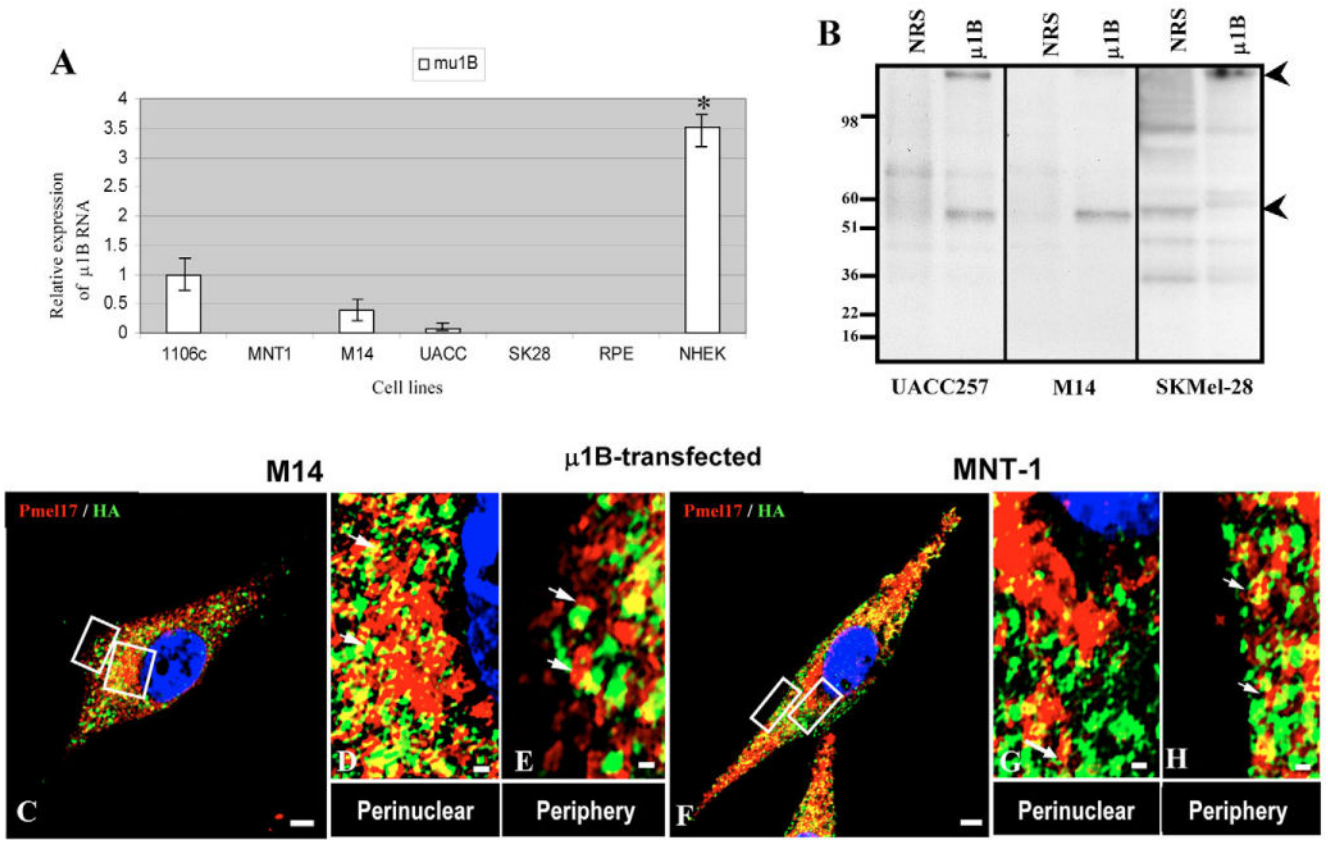

Fig. 4.

Expression of the μ1B subunit in melanocytic cells. (A) μ1B transcript levels determined by semi-quantitative real-time PCR. Note the expression of μ1B in NHEK was significantly higher than in melanocytic cells (*). (B) Melanoma cells as noted were pulse-labeled for 30 minutes, and extracts were immunoprecipitated under denatured and reduced conditions with an anti-μ1B antibody or with normal rabbit serum as a control. Immune complexes were denatured and reduced before separation on gels and visualization by fluorography. (C–H) Cells were fixed 48 hours after transfection with μ1B-HA, and stained with a combination of αPEP13h (red) and anti-HA (green) antibodies (arrows indicate complexes). Specimens were analyzed by confocal microscopy. Nuclei were counterstained with DAPI (blue). Representative images are shown. Bars, 10 μm (C,F); 1 μm (D,E,G,H).

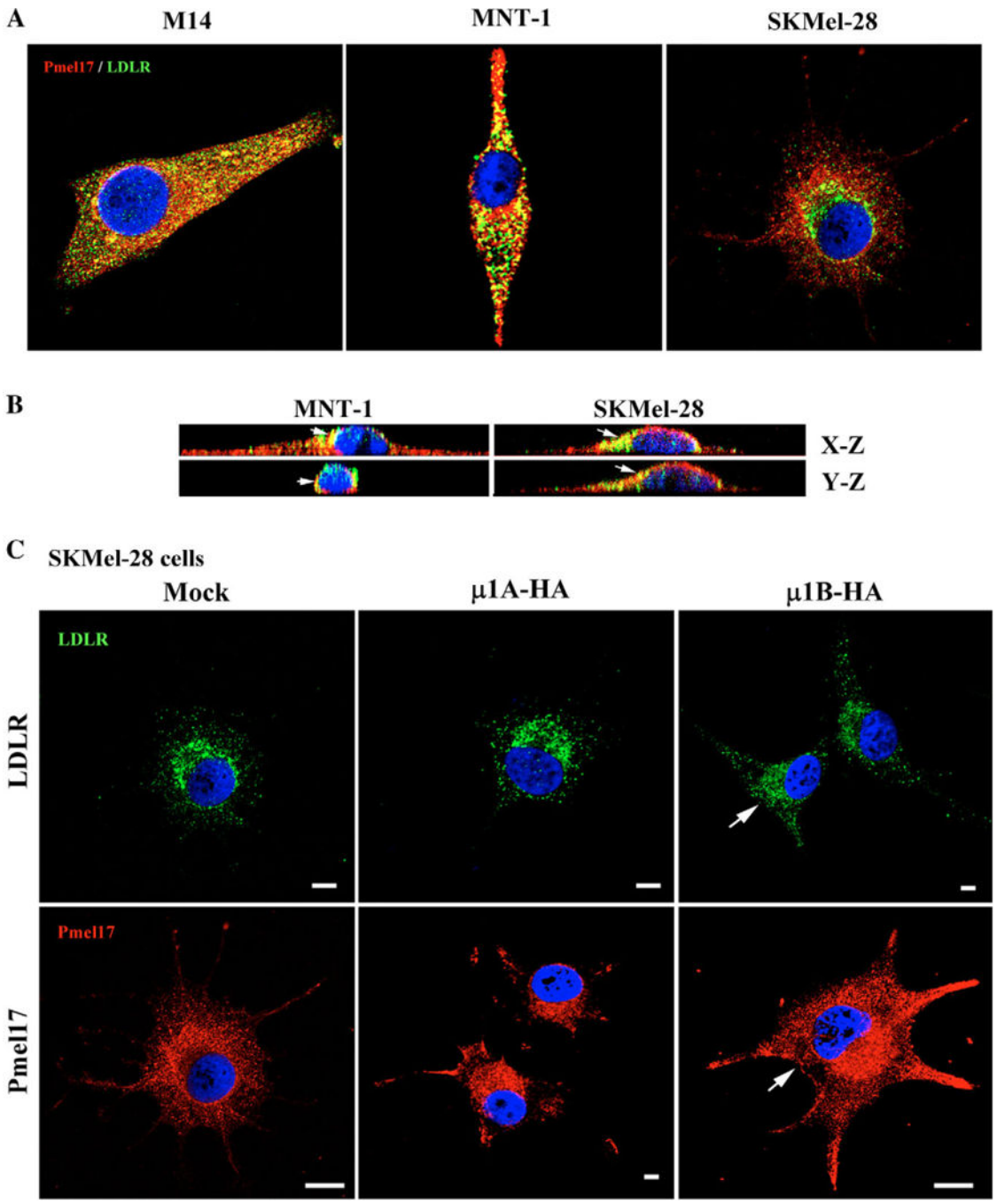

Fig. 5.

Cytoplasmic sorting is restored by µ1B in SKMel-28 cells. (A) M14, MNT-1 and SKMel-28 cells were fixed and double stained for Pmel17 (red) and LDLR (green). Note the restricted distribution of Pmel17 and LDLR in SKMel-28 cells. (B) *Z*-scan analysis shows localization of LDLR with Pmel17 at the upper plasma membrane of MNT1 cells. Note, LDLR and Pmel17 were retained in the perinuclear area (arrows). (C) SKMel-28 cells were transfected with µ1A or µ1B and were cultured for 2 days. Cells were processed for staining of Pmel17 (red) and LDLR (green) by dual immunofluorescence and were analyzed by confocal

microscopy. Note that transfection of μ1B restored cytoplasmic and plasma membrane localization (arrows) for both Pmel17 and LDLR. Representative images from each experiment are shown. Bars, 20 μm.

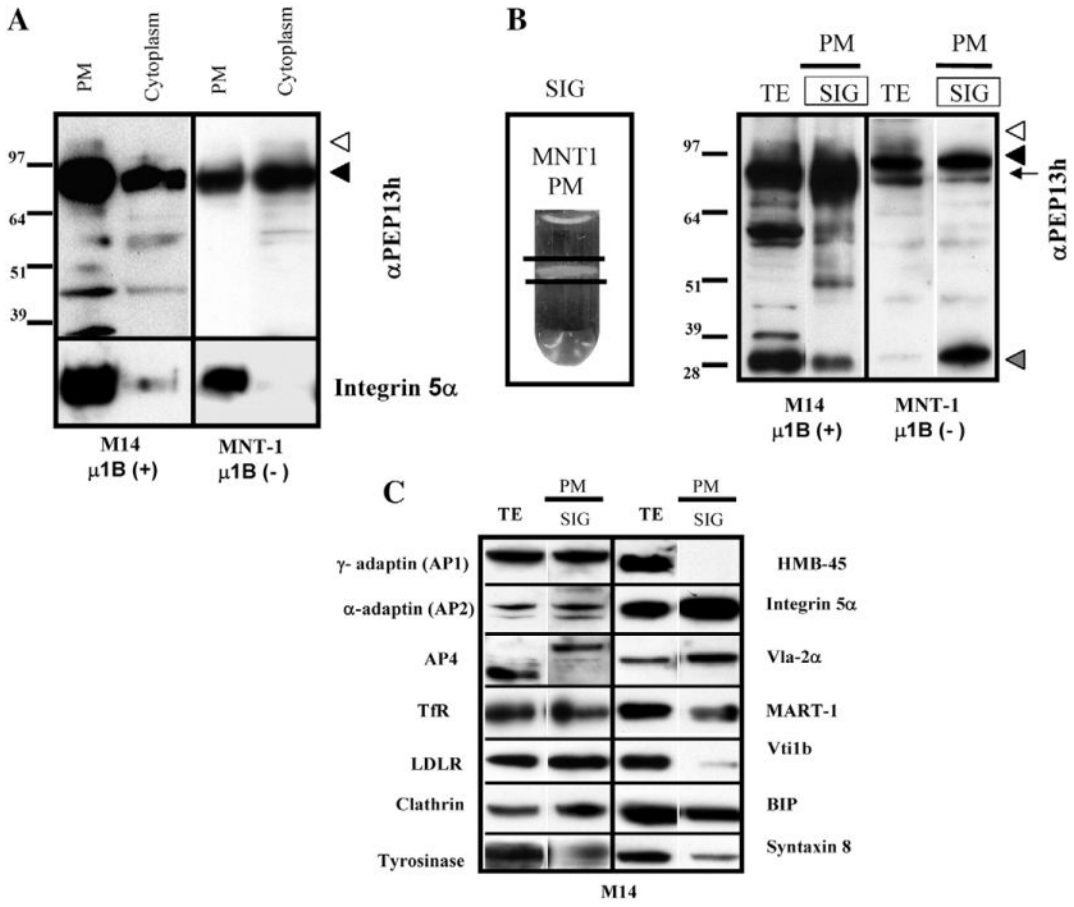

Fig. 6.

Localization of Pmel17 in the plasma membrane. (A,B) Plasma membrane (PM) proteins recovered by crosslinking and SIG purification were analyzed by immunoblotting. All samples were processed in parallel with negative controls. Arrowheads indicate the fully glycosylated ~100 kDa band (white), the partially glycosylated 85 kDa band (black), and the cleaved 26 kDa band of Pmel17 (gray). The black arrow identifies a ~70 kDa band. (A) Pmel17 was recovered using a biotinylated reagent from the plasma membrane in M14 cells and in MNT1 cells. Recovery of the resident plasma membrane protein, I5α, was used as control for the extraction method (see bottom panel). (B) Plasma membrane fraction after SIG purification (left). Using immunoblot analysis, Pmel17 was detected with the αPEP13h antibody both in total extracts (TE) and in the SIG fraction. (C) Plasma membrane fractions were analyzed for the presence of markers as noted. Images are representative of two independent experiments.

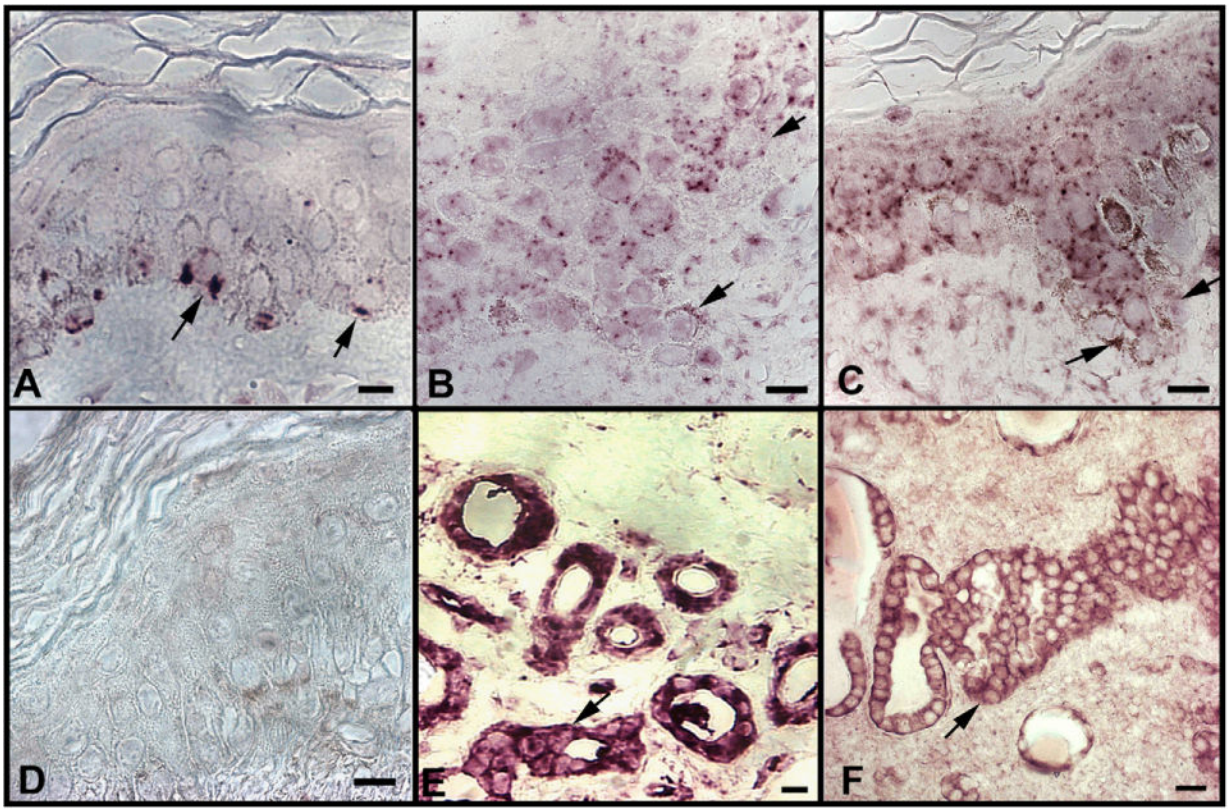

Fig. 7.

Detection of μ1B by TISH in the skin. Skin biopsies were examined by TISH as reported in the Methods. (A,B) Skin before UV stimulation shows Pmel17 and μ1B expression in melanocytes (black arrows) in the epidermis, respectively. (C) Skin 1 day after UV stimulation, shows a moderate increase in the number of μ1B-positive cells and intensity of μ1B expression. (D) Staining with sense probe showed no specific staining. (E,F) Specific expression of μ1B in the cytoplasm of polarized epithelial cells in the skin (sebaceous glands) and kidney (tubular cells) as positive controls. Bars, 100 μm.

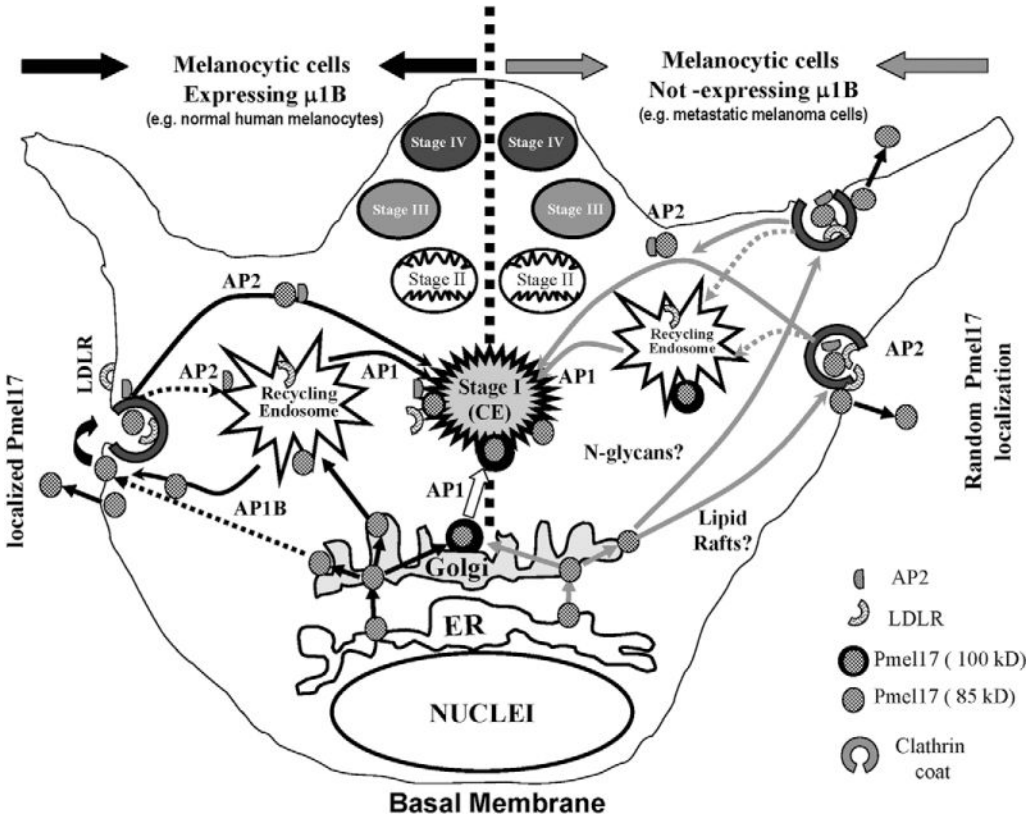

Fig. 8.

Scheme for Pmel17 trafficking. Pmel17 is sorted from the Golgi to stage I melanosomes directly or indirectly according to whether μ1B is expressed. In melanocytic cells expressing μ1B (left side, black arrows), Pmel17 (85 kDa) is sorted from the TGN either to recycled endosomes or directly to a localized region in the plasma membrane via CCV containing μ1B. Pmel17 is then endocytosed and sorted to stage I melanosomes via AP2. In melanocytic cells that do not express μ1B (right side, gray arrows), default sorting of Pmel17 to the entire surface of the plasma membrane may be mediated by glycosylation or by other unknown determinants. Again, endocytosis and sorting to stage I melanosomes is via AP2. In both cases, the mPmel17 (~100 kDa) resulting from its rapid processing and minimal presence in the plasma membrane, is sorted directly to melanosomes, probably via AP1A (white central arrow).

Table 1

Gene profile for AP subunits after treatment of human melanocytes with DKK1

Description*	Fold difference†	DKK1 stimulated	Unstimulated
AP4, β1	2.90	708	244
AP2, α1, transcript variant 2	1.75	13952	7951
GAP3, β2	1.73	2047	1180
AP1, γ1	1.51	9483	6253
AP2, σ1, transcript variant AP17	1.45	114745	78886
AP1, μ2	1.44	1580	1096
AP1, σ3	1.39	1635	1170
AP1, σ1, transcript variant 2	1.38	44659	32257
AP3, μ1, transcript variant 2	1.35	16436	12167
AP2, β1	1.33	45241	34111
AP3, σ1, transcript variant 1	1.15	28072	24332
AP1, β1, transcript variant 2	1.11	6649	5984
AP1, σ2	1.07	170783	159244
AP4, μ1	1.02	3036	2961
AP3, σ2	1.00	4031	4010
AP4, β1	0.97	943	971
AP1, γ2, transcript variant 1	0.87	2070	2386
AP2, μ1	0.80	78539	98451
AP1, μ1	0.78	17410	22235
AP3, σ2	0.78	5378	6894
AP4, ε1	0.70	1225	1747
AP3, μ2	0.69	16251	23540
AP4, σ1	0.69	792	1153
AP2, α2	0.66	4517	6885
AP3, β1	0.61	11335	18478
AP3, δ1	0.57	43877	76592

*Greek characters indicate AP family subunits.

†Values above 1.4 are considered upregulated; values below 0.7 are considered downregulated.