

Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis

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Lysosome-related organelles are cell type-specific intracellular compartments with distinct morphologies and functions. The molecular mechanisms governing the formation of their unique structural features are not known. Melanosomes and their precursors are lysosome-related organelles that are characterized morphologically by intraluminal fibrous striations upon which melanins are polymerized. The integral membrane protein Pmel17 is a component of the fibrils and can nucleate their formation in the absence of other pigment cell-specific proteins. Here, we show that formation of intraluminal fibrils requires

cleavage of Pmel17 by a furin-like proprotein convertase (PC). As in the generation of amyloid, proper cleavage of Pmel17 liberates a luminal domain fragment that becomes incorporated into the fibrils; longer Pmel17 fragments generated in the absence of PC activity are unable to form organized fibrils. Our results demonstrate that PC-dependent cleavage regulates melanosome biogenesis by controlling the fibrillogenic activity of a resident protein. Like the pathologic process of amyloidogenesis, the formation of other tissue-specific organelle structures may be similarly dependent on proteolytic activation of physiological fibrillogenic substrates.

Introduction

Organelle function and morphology are intimately related, yet the molecular mechanisms that underlie organelle morphogenesis are largely uncharacterized. Organelle morphology within the central vacuolar system is controlled by extrinsic factors, such as cytoskeletal or “matrix” proteins (De Matteis and Morrow, 2000; Warren and Shorter, 2002), and by intrinsic factors, such as resident integral membrane and luminal contents (Valladeau et al., 2000). Organelle morphogenesis must be regulated to ensure proper temporal and spatial control; dysregulation may result in disease, as exemplified by the defects in lysosome-related organelles in pa-

tients with Hermansky-Pudlak Syndrome and corresponding mouse models (Swank et al., 1998; Spritz, 1999; Dell’Angelica et al., 2000). Understanding how morphogenesis of lysosome-related organelles is controlled may help to elucidate the etiology of these disorders.

Melanosomes serve as an excellent model to define biogenetic mechanisms among lysosome-related organelles. Melanosomes are specialized organelles within melanocytes and retinal pigment epithelial cells in which melanin pigments are generated and stored (Marks and Seabra, 2001; Raposo and Marks, 2002). As they are synthesized within maturing (stage III and IV) melanosomes, melanins are deposited along characteristic intraluminal fibers that appear before pigment deposition in stage II premelanosomes. Like the dense cores of secretory granules, the fibers serve to sequester and concentrate cargo—in this case melanins; they also likely function to detoxify oxidative melanin intermediates (Seiji et al., 1963; King et al., 1995). The fibers are thought to be proteinaceous because they lack the ultrastructural hallmarks of a lipid bilayer (Moyer, 1966; Maul, 1969; Hearing et al., 1973). Thus, they are similar to intraluminal fibrils that form in certain pathologies associated with amyloid deposition (Badman et al., 1996; Kim et al., 1999; Chen et al., 2001). The formation of fibrils represents a key

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*Abbreviations used in this paper: α_1 -AT, α_1 -antitrypsin inhibitor; α_1 -PDX, α_1 -antitrypsin Portland; endoH, endoglycosidase H; IEM, immunoelectron microscopy; IFM, immunofluorescence microscopy; ILV, intraluminal vesicle; moi, multiplicity of infection; MVB, multivesicular body; PC, proprotein convertase; tTA, tetracycline-repressible transactivator; TX, Triton X-100.

Key words: furin; endosome; proteolysis; fibril; lysosome-related organelle

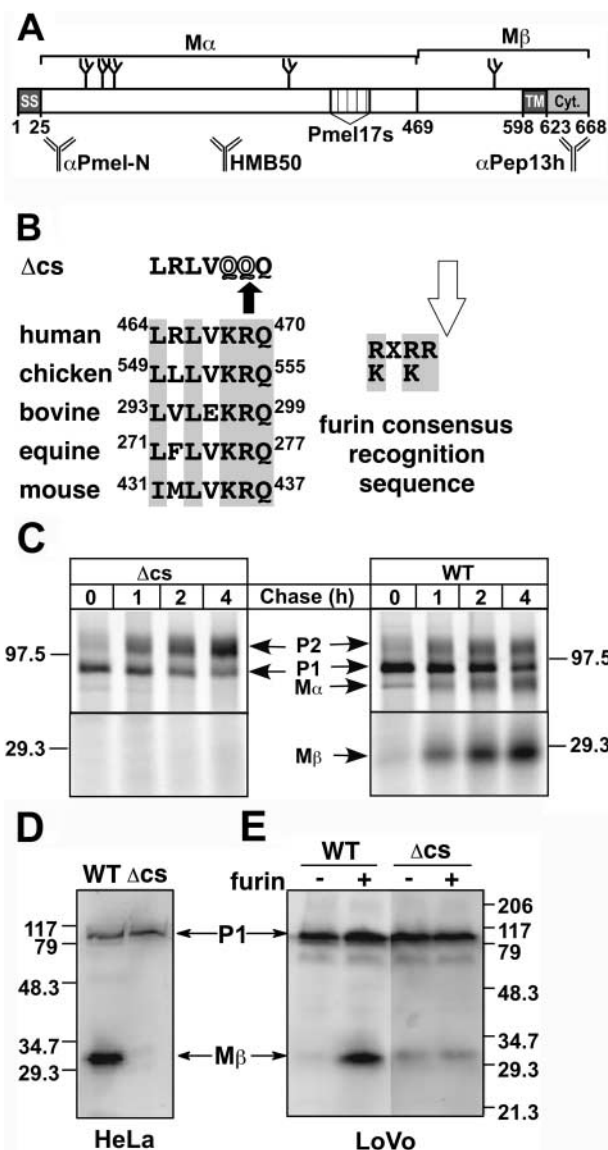


Figure 1. Identification of a conserved PC cleavage site in Pmel17. (A) Schematic diagram of the primary structure of Pmel17 drawn to scale. Numbers correspond to amino acid positions; positions of the signal sequence (SS), transmembrane (TM), and cytoplasmic (Cyt.) domains, aa 469, and a luminal region deleted in the alternative spliced form, Pmel17-s, are indicated; N-linked glycans are represented by branched structures; regions corresponding to M α and M β are shown above; and regions recognized by antibodies α Pmel-N, HMB50, and α Pep13h are shown below. (B) Sequence of the conserved dibasic consensus site from orthologues human Pmel17 (GenBank/EMBL/DDBJ accession no. NP_008859), chicken MMP115 (GenBank/EMBL/DDBJ accession no. Q98917), bovine RPE1 (GenBank/EMBL/DDBJ accession no. Q06154), equine Pmel17 (GenBank/EMBL/DDBJ accession no. AAC97108), and mouse Silver (GenBank/EMBL/DDBJ accession no. NP_068682). Amino acid positions are noted (equine and bovine sequences are from incomplete clones); conserved residues are in gray. Top, Pmel17 Δ cs (Δ cs) sequence. Right, consensus recognition sequence for furin; cleavage site is indicated by arrow. (C) Cleavage is inhibited by mutagenesis of the dibasic site. Transiently transfected HeLa cells expressing wild-type Pmel17 (WT) or Pmel17 Δ cs (Δ cs) were metabolically pulse labeled and chased as indicated. TX cell lysates at each time point were immunoprecipitated with α PEP13h, fractionated by SDS-PAGE, and analyzed by phosphorimaging. Shown are regions of the gels encompassing the relevant bands; the signal from the lower region

step in melanosome biogenesis, but their composition and the molecular mechanisms underlying their formation are unknown.

Pmel17 (also known as gp100, ME20, and Silver) is a type I integral membrane protein, uniquely expressed by melanocytes and retinal pigment epithelial cells, that localizes to the premelanosome matrix (Kwon et al., 1987; Vennegoor et al., 1988; Adema et al., 1994; Kobayashi et al., 1994; Zhou et al., 1994; Lee et al., 1996; Raposo et al., 2001). A role for Pmel17 in melanosome function is supported by the pigmentation defect in *silver* mice (Dunn and Thigpen, 1930), in which the Pmel17 protein is truncated (Martínez-Esparza et al., 1999). Pmel17 immunolocalizes to the fibrils within stage II premelanosomes (Berson et al., 2001; Raposo et al., 2001), and Pmel17 expression is sufficient to induce the formation of premelanosome-like fibrils within multivesicular bodies (MVBs)* of nonpigment cells (Berson et al., 2001). Nevertheless, Pmel17 in nondenaturing detergent extracts of melanocytes or transfected HeLa cells remains covalently tethered to its transmembrane domain, even after the precursor polypeptide is cleaved into two fragments (Berson et al., 2001). This implies a continued association with membranes, belying the inferred non-membranous nature of the striations. To probe the mechanism by which Pmel17 initiates fibril formation, we asked (1) what is the function of the intraluminal cleavage; and (2) what is the biochemical nature of Pmel17 on the intraluminal striations? Our results highlight a role for proprotein convertases (PCs) in organelle biogenesis, and reveal a striking parallel between the formation of the morphological hallmark of premelanosomes and the pathological fibril formation in amyloid disease.

Results

Identification of Pmel17 cleavage site and proteolysis by a PC

In human melanocytes, melanoma cells, and transfected nonpigment cells, Pmel17 is proteolytically cleaved within a post-Golgi compartment (Berson et al., 2001). Intraluminal cleavage generates a COOH-terminal 28-kD M β fragment containing part of the luminal domain and the entire transmembrane and cytoplasmic domains, and an \sim 80-kD M α fragment consisting of the remaining NH₂-terminal region of the luminal domain (Fig. 1 A). Both fragments are stoichiometrically coprecipitated from cell lysates prepared with Triton X-100 (TX); a small amount of M α , free of M β , is precipitated from cell supernatants (Berson et al., 2001). M α migrates identically to ME-20s, consisting of

was enhanced fourfold to emphasize the failure to detect M β from Pmel17 Δ cs. Migration of molecular weight markers and P1, P2, M α , and M β forms of Pmel17 are indicated. (D and E) Western blot analysis of wild-type Pmel17 (WT) and Pmel17 Δ cs (Δ cs) expressed transiently by transfection in (D) HeLa or (E) LoVo cells. In E, LoVo cells were cotransfected (+) or not (-) with a plasmid encoding rat furin. Whole-cell lysates from transfected cells were fractionated by SDS-PAGE, transferred to PVDF membranes, and probed using α PEP13h with enhanced chemifluorescence. Positions of molecular weight markers and P1 and M β forms of Pmel17 are indicated.

Pmel17 residues 25–467, identified in melanoma cell supernatants (Maresh et al., 1994).

Immediately after V⁴⁶⁷ in Pmel17 is a conserved dibasic pair within a consensus sequence that is similar to the recognition element for PCs of the furin family of proteases (Fig. 1 B; Hosaka et al., 1991). PCs and associated carboxypeptidases mediate regulated cleavage of proproteins within late secretory compartments and endosomes (Steiner, 1998; Varlamov and Fricker, 1998; Thomas, 2002). ME20s/M α could potentially be generated by PC-dependent cleavage at R⁴⁶⁹ followed by removal of the dibasic pair by a carboxypeptidase D- or E-like activity. To determine whether Pmel17 cleavage requires the dibasic pair, KR^{468–469} was altered to QQ by site-directed mutagenesis, generating Pmel17 Δ cs (Fig. 1 B). Unlike wild-type Pmel17, Pmel17 Δ cs expressed in transfected HeLa cells was not processed to M α and M β (Fig. 1, C and D). Instead, cells accumulated the P2 form, representing full-length Pmel17 Δ cs with Golgi-modified N-linked glycans (Berson et al., 2001). Furthermore, although cells expressing wild-type Pmel17 secrete low levels of M α into the medium, cells expressing Pmel17 Δ cs did not (unpublished data).

To determine whether PCs are required for the cleavage, Pmel17 or the Δ cs variant were expressed in LoVo, a human adenocarcinoma cell line lacking furin (Takahashi et al., 1995) and several other PCs (Miranda et al., 1996). Cleavage in transfected LoVo or control HeLa cells was assessed by Western blotting of whole-cell lysates with α PEP13h, an antibody to the cytoplasmic domain. Lysates from HeLa cells expressing wild-type Pmel17, like melanocytic cells, contain two immunoreactive bands with M_r 28,000 and \sim 100,000, representing M β and the full-length, endoglycosidase H (endoH)-sensitive P1 precursor, respectively (Fig. 1 D; see Fig. 5 A). By contrast, in both HeLa cells expressing Pmel17 Δ cs (Fig. 1 D) and LoVo cells expressing wild-type Pmel17 (Fig. 1 E), only the \sim 100-kD P1 band and not M β was observed, indicating that cleavage did not occur (a faint residual band that co-migrates with M β in LoVo cells expressing Pmel17 Δ cs may result from cleavage by NARC-1, a related protease that is active within the ER and for which a sequence similar to the consensus recognition site is generated by the KR^{468–469} \rightarrow QQ mutation; Seidah et al., 2003). M β was restored in LoVo cells by coexpression of both Pmel17 and furin, but not Pmel17 Δ cs and furin (Fig. 1 E). Thus, furin or a similar PC can cleave Pmel17 at the dibasic recognition site. Importantly, cleavage at the only other dibasic sites in Pmel17, KR¹⁴⁷, or RR¹⁹², would not generate fragments of the appropriate size for M α and M β .

To determine whether a furin-like PC cleaves Pmel17 in melanocytic cells, we inhibited PCs in the pigmented human melanoma, MNT-1, by expressing α_1 -antitrypsin Portland (α_1 -PDX), a PC-specific variant of α_1 -antitrypsin inhibitor (α_1 -AT; Anderson et al., 1993; Jean et al., 1998, 2000). FLAG epitope-tagged α_1 -PDX or α_1 -AT were expressed in MNT-1 cells by infection with recombinant adenoviruses. Transgene expression and Pmel17 processing were assessed by metabolic pulse/chase, immunoprecipitation, SDS-PAGE, and phosphorimaging analysis. Both transgenes were efficiently expressed, although a lower mul-

tiplicity of infection (moi) with the α_1 -PDX virus was required to obtain the same level of expression as α_1 -AT (Fig. 2 A; likely due to contaminating nonrecombinants in the α_1 -AT preparation). As described previously, Pmel17 in uninfected cells first appears as endoH-sensitive P1 and Pmel17-s forms (Berson et al., 2001). P1 is the full-length core glycosylated precursor, and Pmel17-s is the product of an alternatively spliced mRNA that is generated in melanocytic cells (Nichols et al., 2003), but not transfected HeLa cells (Berson et al., 2001); Pmel17-s is distinguished from M α based on its direct reactivity with α Pep13h, its appearance in the pulse-labeled samples, and its sensitivity to EndoH (see Fig. 5). P1 and Pmel17-s are subsequently modified in the Golgi to endoH-resistant P2, and P2 is then cleaved to M α and M β (Fig. 2 B, lanes 1–5). All fragments disappeared from detergent cell lysates with a half-life of 2.5 h. In cells expressing α_1 -AT, the pattern and kinetics of Pmel17 processing were virtually unchanged (Fig. 2 B, lanes 6–10). However, in cells expressing α_1 -PDX, as in HeLa cells expressing Pmel17 Δ cs, P2 accumulated, and generation of M α /M β was inhibited by at least 90% (Fig. 2 B, lanes 11–20; Fig. 2 C). Cleavage was equally inhibited in cells expressing two different levels of α_1 -PDX (Fig. 2, B and C), likely reflecting the secretion of α_1 -PDX by infected cells and uptake by uninfected cells. By contrast, α_1 -PDX did not affect the maturation and half-life of another melanosome protein, Tyrp1 (unpublished data). These data confirm that furin or a furin-like PC mediates Pmel17 cleavage in melanocytic cells.

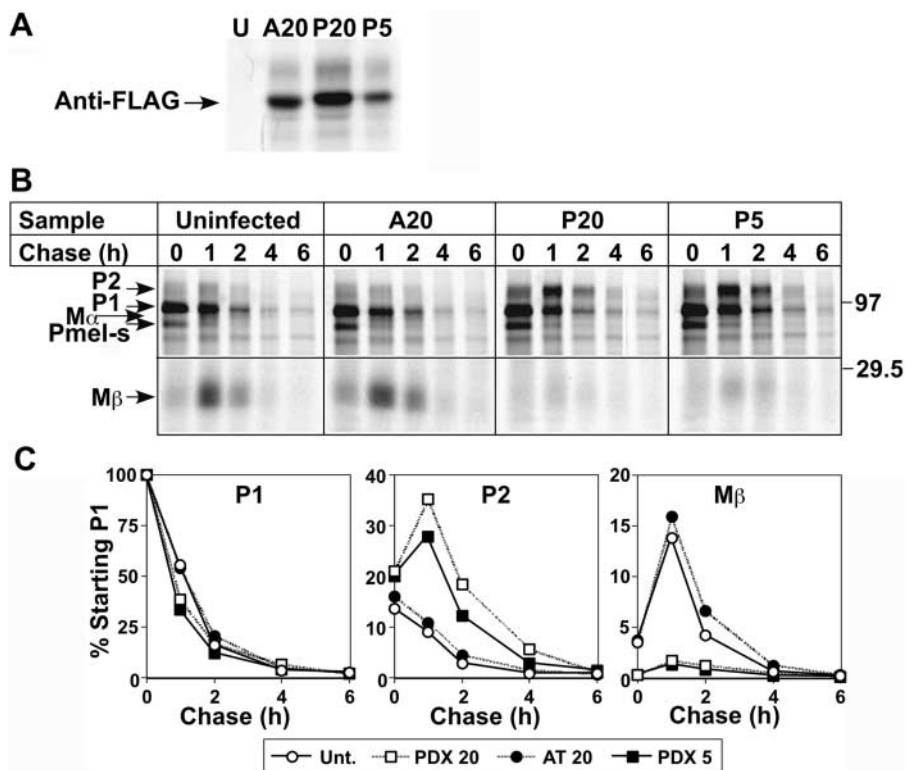
PC cleavage is not required for localization of Pmel17 to multivesicular endosomes

Pmel17 transits through a multivesicular coated endosome en route to striated stage II premelanosomes in pigmented cells (Raposo et al., 2001). Similarly, Pmel17 in transfected HeLa cells localizes mainly to intraluminal vesicles (ILVs) of MVBs (Berson et al., 2001). To determine whether this localization step requires PC-dependent cleavage, HeLa cells expressing wild-type Pmel17 or Pmel17 Δ cs were analyzed by immunofluorescence microscopy (IFM) and immunoelectron microscopy (IEM). Pmel17 Δ cs, like wild-type Pmel17 (Berson et al., 2001), localized by IFM to vesicles that overlapped with a large fraction of vesicles that contained the late endosome/lysosome marker, LAMP1 (unpublished data). IEM of ultrathin cryosections identified these structures as MVBs, in which Pmel17 Δ cs accumulated largely in the ILVs (Fig. 3 A). Similarly, Pmel17 localization to multivesicular-coated endosomes in MNT-1 melanoma cells was not affected by expression of α_1 -PDX (Fig. 4 E). Together with the normal half-life in TX cell lysates and kinetics of conversion from P1 to P2 (Fig. 1 C and Fig. 2 B), these data indicate that ER export and post-Golgi trafficking of Pmel17 are not dramatically influenced by loss of PC cleavage.

Formation of fibrous striations requires PC cleavage

Structures similar to the fibrous striations of premelanosomes are frequently observed in MVBs of transfected HeLa cells that express high levels of wild-type Pmel17 (Berson et al., 2001). By contrast, such structures were not observed in HeLa cells

Figure 2. PCs mediate Pmel17 cleavage in melanocytic cells. MNT-1 cells were uninfected (U) or infected with recombinant adenoviruses encoding tTA (moi of 20) and either FLAG-tagged α_1 -AT (moi of 20; A20) or α_1 -PDX at moi of 20 (P20) or 5 (P5). Two days after infection, cells were metabolically pulse labeled and chased for the indicated periods of time. Immunoprecipitates from TX cell lysates at indicated time points were fractionated by SDS-PAGE and analyzed by phosphorimaging. (A) Epitope-tagged α_1 -AT and α_1 -PDX immunoprecipitated from pulse-labeled cell lysates using an anti-FLAG antibody. Similar levels of transgene expression were obtained using α_1 -PDX at moi of 5 and α_1 -AT at moi of 20. (B) Pmel17 immunoprecipitated using α PEP13h. Only the relevant portion of the gels are shown; no other specific bands were consistently observed. The intensity of the lower part of the gel was amplified 2 \times to emphasize the M β band. Positions of molecular weight markers (right) and of P1, Pmel17-s, P2, M α , and M β (left) are noted. Pmel17-s is the product of an alternatively spliced Pmel17 mRNA. (C) Quantitation of the signal intensity of P1, P2, and M β from B. Data represent the percentage of signal from each band relative to the values for P1 at 0 chase for each group (average from three experiments). Note that the predicted cysteine and methionine content of M β represents 42% of that present in full-length Pmel17.



expressing comparable or higher levels of Pmel17 Δ cs (Table I and Fig. 3 E). Instead, Pmel17 Δ cs accumulated in irregular electron-dense aggregates within compartments that contained intraluminal membrane sheets rather than vesicles, and that thus resembled lysosomes (Fig. 3, B and E). Accordingly, these compartments labeled densely for LAMP1 by IEM (unpublished data). These data suggest that if Pmel17 is not cleaved by PCs, its ability to nucleate striation formation is inhibited concomitant with enhanced movement from late endosomes to lysosomes. The dense aggregates observed by IEM may represent intermediates in the degradation of Pmel17 by lysosomal proteases (see Fig. 4 and Fig. 5).

To determine whether Pmel17 cleavage was required for striation formation in melanocytic cells, MNT-1 cells expressing α_1 -PDX or α_1 -AT were analyzed by EM (Fig. 4).

Table I. Quantitation of Pmel17 localization to MVBs and striations in transfected HeLa cells

	HeLa Pmel17 WT	HeLa Pmel17 Δ cs
MVBs	57%	51%
MVBs + striations	43%	2% ^a
Lysosomes + aggregates	–	47%

HeLa cells expressing Pmel17 WT or Pmel17 Δ cs were fixed, and ultrathin cryosections were labeled with HMB50 anti-Pmel17 antibody and PAG-10. 50 cell profiles of each were analyzed for the presence of gold labeling in MVBs or lysosomes, and for the association of the gold particles with striation-like structures or aggregates. Percentages are relative to the total number of structures analyzed in all cell profiles (150 compartments for WT, 160 for Δ cs).

^aThese correspond to MVBs with non-organized proteinaceous material.

Because α_1 -PDX or α_1 -AT are secreted, even uninfected cells in the cultures were partially subject to their effects. Although expression of α_1 -AT had little effect on the abundance or morphology of melanosomes or on the localization of Pmel17 (Fig. 4, A and B), many cells expressing α_1 -PDX were depleted of striated stage II premelanosomes, and most accumulated irregular pigment-containing structures (Fig. 4, C–E). Some of these structures were multivesicular (Fig. 4 D), and others contained irregular melanin aggregates (Fig. 4 C). Ultrastructurally normal stage II, III, and IV melanosomes in these cells were reduced in number; quantitative analyses of 50 cell profiles in Epon sections indicated that 16% lacked any stage II premelanosomes, and an additional 72% had disorganized melanosome structures. By IEM, Pmel17 in these cells accumulated in membrane-enclosed structures with disordered aggregates of fuzzy electron-dense material, similar to those observed in Pmel17 Δ cs-expressing HeLa cells (compare Fig. 4 A with Fig. 4 E); these likely represent malformed stage II premelanosomes, late endosomes, or lysosomes with mistargeted Pmel17. By contrast, Pmel17 labeling in both the limiting and intraluminal membranes of coated endosomes in MNT-1 cells was not affected by α_1 -PDX expression (Fig. 4 E). These results confirm the importance of Pmel17 cleavage in the formation of characteristic intraluminal striations of melanosomes.

Association of the luminal domain fragment with TX-insoluble complexes

The dependence of striation formation on PC cleavage of Pmel17 would support a model in which M α fragments

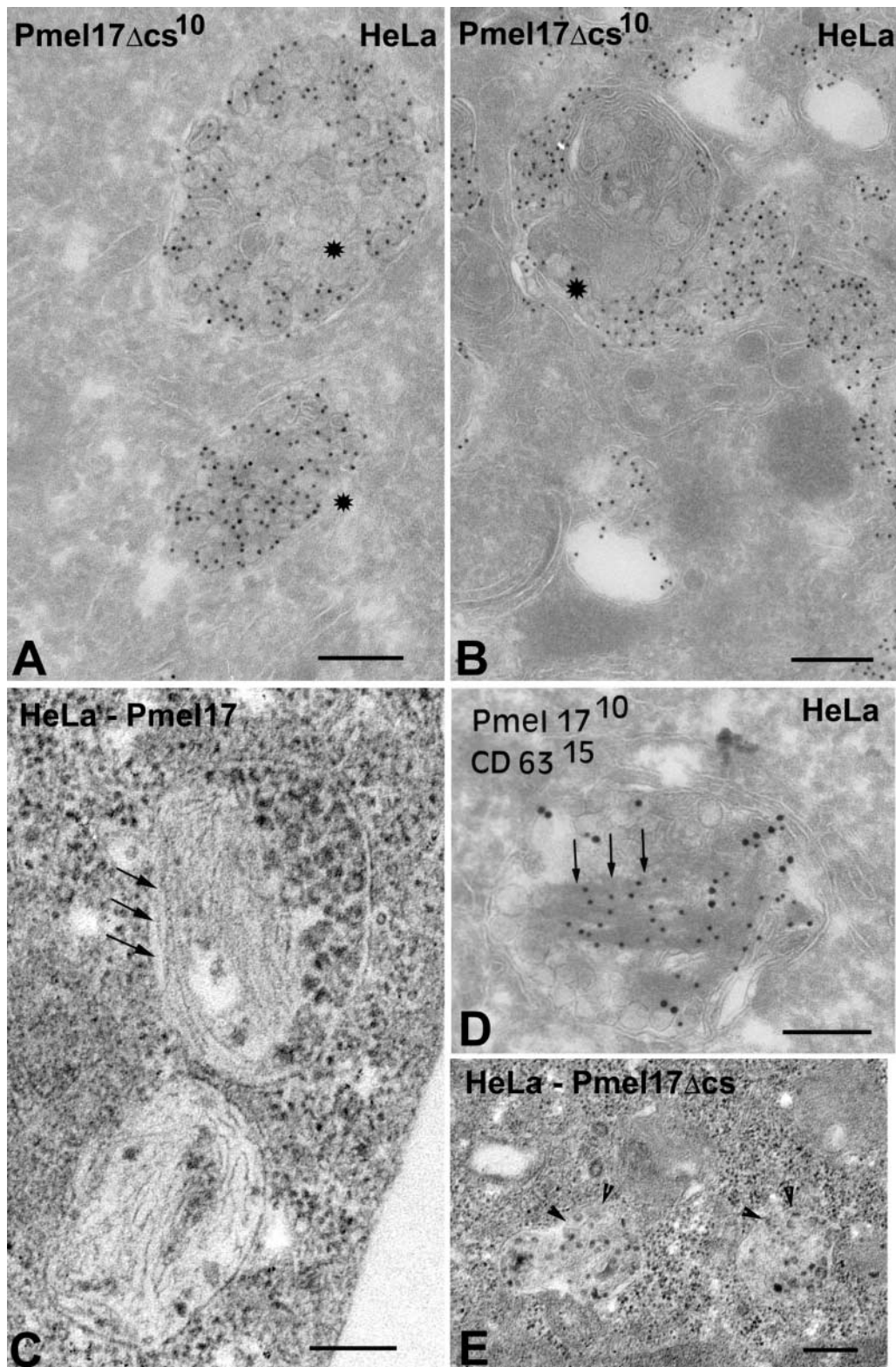


Figure 3. **Cleavage site-deficient Pmel17 localizes to MVBs, but fails to form fibers.** Transiently transfected HeLa cells expressing Pmel17 Δ cs¹⁰ (A, B, and E) or wild-type Pmel17 (C and D) were analyzed by either IEM (A, B, and D) or standard EM (C and E). (A, B, and D) Ultrathin cryosections were immunogold labeled with HMB50 and PAG-10; in D, sections were double labeled with anti-CD63 and PAG-15. (A) A typical Pmel17 Δ cs-expressing cell showing predominant labeling on ILVs of multivesicular late endosomes. (B) A cell with very high Pmel17 Δ cs expression; note the gold labeling over electron-dense aggregates in compartments with multiple layers of membranes (stars). (D) A cell with high wild-type Pmel17 expression; note the gold labeling over ILVs and fibrous structures within MVBs (arrows), and exclusion of CD63 labeling from the striated regions. (C) Standard EM of a cell expressing wild-type Pmel17. Note the fibers within MVBs (arrows). (E) Standard EM of a cell expressing Pmel17 Δ cs. Note the accumulation of electron-dense material in MVBs, but no obvious fibers (arrowheads). Bars, 200 nm.

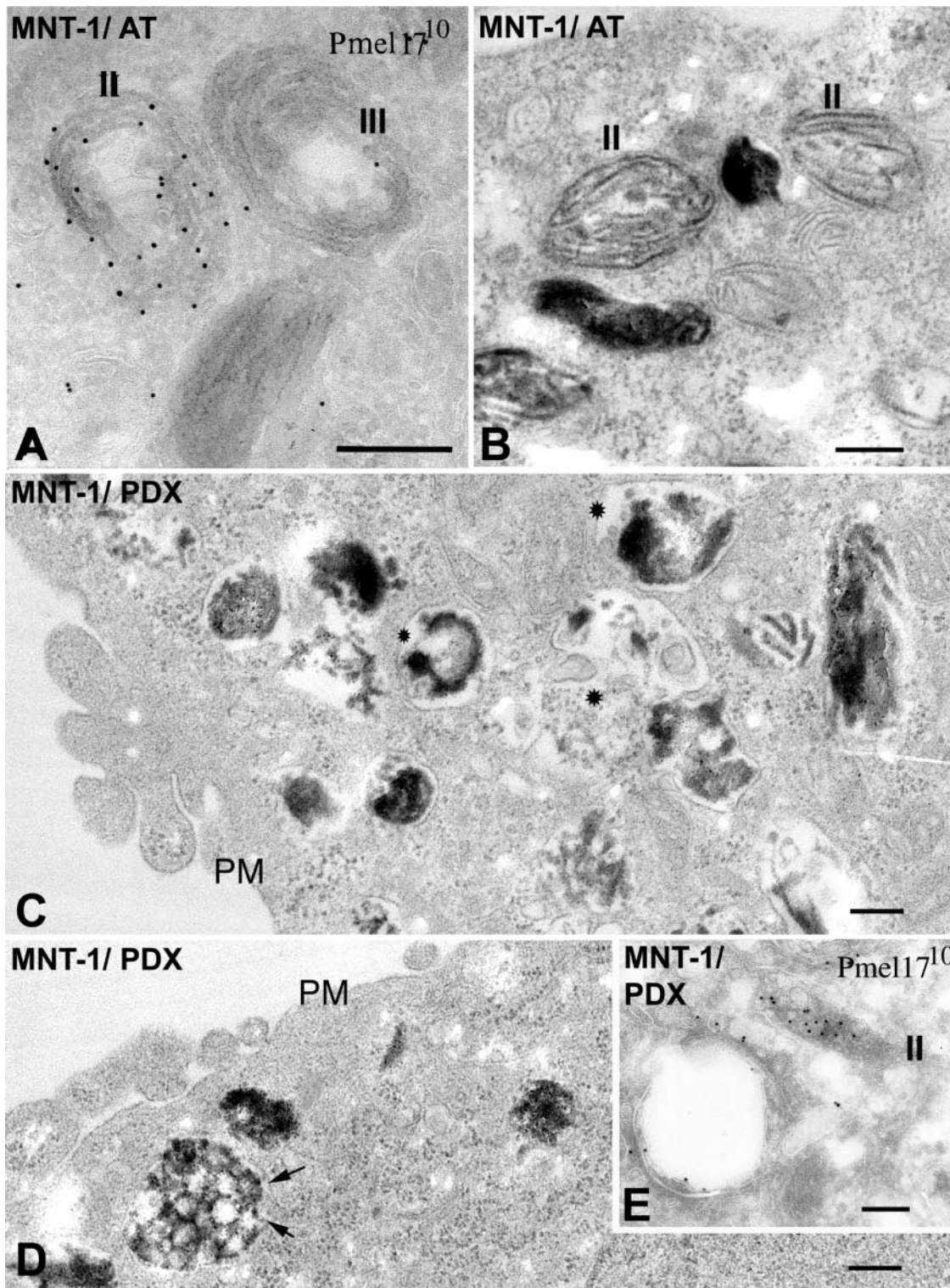


Figure 4. Disruption of melanosome morphology in MNT-1 cells expressing α_1 -PDX. MNT-1 cells infected with recombinant adenoviruses expressing tTA and either α_1 -AT (A and B) or α_1 -PDX (C–E) were analyzed by either IEM (A and E) or standard EM (B–D). (A and E) Ultrathin cryosections were immunogold labeled with HMB50 and PAG-10. (A and B) Note the characteristic striations of stage II and III premelanosomes (II and III) in cells expressing α_1 -AT. By IEM, these structures are densely labeled for Pmel17. (C and D) Note the absence of normal stage II and III premelanosomes in cells expressing α_1 -PDX, and the appearance of unusual melanin aggregates in disordered arrays (C, stars) and multivesicular structures (D, arrows). (E) By IEM, Pmel17 is detected normally in endosomes with a planar clathrin coat (bottom left), but dense labeling is limited to abnormal, nonstriated, multivesicular structures with the shape of stage II premelanosomes (II). PM, plasma membrane. Bars, 200 nm.

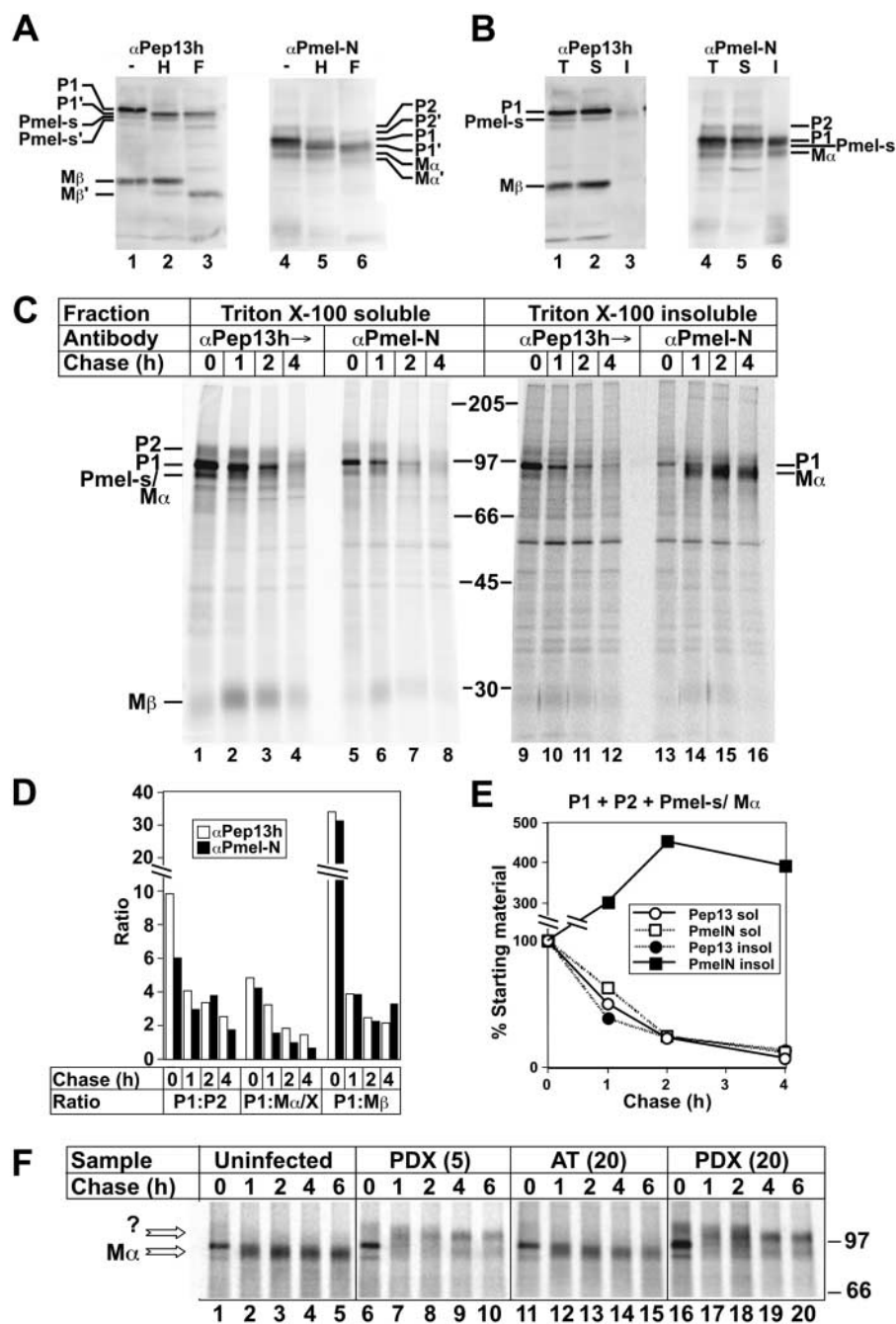


Figure 5. M α accumulation in TX-insoluble fractions of MNT-1 cells and inhibition by α_1 -PDX. (A and B) Western blot analyses of MNT-1 cell fractions. Lysates fractionated by SDS-PAGE were probed with antibodies α Pep13h (to COOH terminus) or α Pmel-N (to NH₂ terminus). (A) Whole-cell lysates were untreated or treated with endoH (H) or N-glycanase F (F). (B) Whole-cell lysates (T) and TX-soluble (S) or -insoluble (I) material were analyzed directly. Relevant bands are indicated; bands annotated with ' are glycosidase cleavage products. (C-F) Pulse-chase/immunoprecipitation analyses of M α formation in MNT-1 cells. Metabolically pulse-labeled and chased MNT-1 cells were extracted with TX, and TX-insoluble pellets were re-extracted in lysis buffer containing 8M urea at 60°C. TX-soluble and -insoluble/urea extracted fractions (after dilution of the urea) were sequentially immunoprecipitated first with normal rabbit serum, then with α PEP13h, and finally with α Pmel-N. Immunoprecipitates were fractionated by SDS-PAGE and analyzed by phosphorimaging. Chase times (h) are indicated at top (C and F) or bottom (D and E); migration of molecular weight markers and relevant bands are indicated to the side. (C) α Pep13h and α Pmel-N immunoprecipitates from both fractions are shown. Panels on the right were exposed 22 \times longer than those on the left because of vastly reduced efficiency of immunoprecipitation after treatment of lysates with urea (unpublished data). Note the appearance of M α in α Pmel-N immunoprecipitates at later time points. (D) The intensity of indicated bands in each lane from the soluble fraction of C was quantitated by phosphorimaging, and ratios of the intensities of P1 to P2, Pmel17-s/M α (M α /X), and M β at each time point, precipitated by either α Pep13h or α Pmel-N, were calculated and plotted; the Pmel17-s and M α bands were summed because they could not be easily resolved. Note that for each time point, ratios were similar for both antibodies. (E) Intensities

of P1, P2, Pmel17-s, and M α bands were summed at each time point for both antibodies in both soluble (sol) and insoluble (insol) fractions, and plotted as a percentage of the value at time 0. Note the loss of material from all fraction/antibody combinations except the insoluble fraction precipitated with α Pmel-N, in which intensity increased over time. (F) Cells were uninfected or infected with recombinant adenoviruses expressing α_1 -PDX at moi of 5, PDX(5); or 20, PDX(20); or α_1 -AT at moi of 20, AT(20), before the experiment; shown are only the relevant regions of the α Pmel-N immunoprecipitates from the TX insoluble fraction. Arrows, bands that co-migrate with M α or a novel product (?) precipitated only from cells expressing α_1 -PDX.

form intraluminal proteinaceous fibrils upon their release from membranes. By analogy to amyloid, such fibrils might be insoluble in nondenaturing detergents such as TX. Indeed, M α was observed in TX-insoluble fractions of MNT-1 cells. By Western blotting, an endoH-resistant, N-glycanase F-sensitive M α band was detected in whole MNT-1 cell lysates with α Pmel-N (antibody to the lumenally exposed NH₂ terminus of Pmel17), but not with α Pep13h (antibody to the cytoplasmically exposed COOH terminus;

Fig. 5 A), distinguishing it from the endoH-sensitive, α Pep13h-reactive alternative splice product, Pmel17-s. Although most M β , Pmel17-s, and P1 isoforms were soluble in TX, a significant fraction of M α was detected in the TX-insoluble pool (Fig. 5 B).

Formation of TX-insoluble M α was kinetically linked to both cleavage and dissociation from M β by a metabolic pulse/chase assay in which TX-insoluble material from cell lysates was extracted by heating in 8M urea. Pmel17 iso-

forms from this TX-insoluble pool (after dilution of the urea) and from the soluble fraction were sequentially immunoprecipitated with α PEP13h and then α Pmel-N, then analyzed by SDS-PAGE and phosphorimaging. From the soluble pool, α Pmel-N only immunoprecipitated residual P1, P2, Pmel17-s, $M\alpha$, and $M\beta$ in similar proportions as in the initial α Pep13h immunoprecipitates (Fig. 5, C and D; compare lanes 1–4 with lanes 5–8). From the TX-insoluble pool, although α Pep13h immunoprecipitated only full-length P1 and Pmel17-s (Fig. 5 C, lanes 9–12), subsequent α Pmel-N immunoprecipitates additionally contained an \sim 80-kD band (lanes 13–16); its failure to be depleted by α Pep13h suggests that this band is $M\alpha$. The intensity of this band increased over time from undetectable at the pulse to most intense by 4 h of chase (Fig. 5, C and E). Thus, at least a portion of $M\alpha$ was released from $M\beta$ during the chase and accumulated in TX-insoluble complexes.

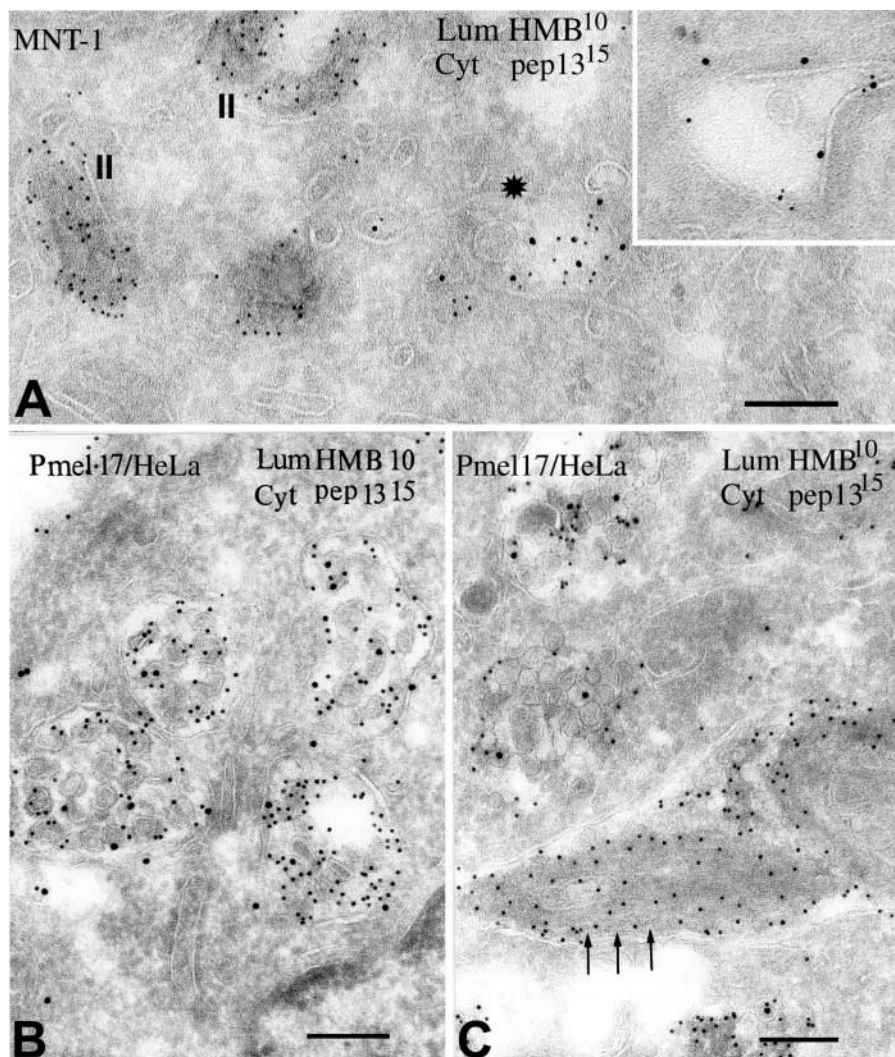
To confirm the identity of this TX-insoluble band as $M\alpha$ and to determine whether its accumulation required PC cleavage, MNT-1 cells expressing α_1 -PDX were similarly analyzed by pulse/chase and immunoprecipitation. As shown in Fig. 5 F, expression of α_1 -PDX (but not α_1 -AT) inhibited the formation of TX insoluble $M\alpha$. Appearance of the frag-

ment was both delayed and reduced in efficiency by expression of α_1 -PDX. Instead, these cells accumulated a larger fragment of Pmel17, lacking the COOH terminus and possibly comprising the entire luminal domain (Fig. 5 F; band marked “?”). Appearance of this band correlates with the disordered aggregates observed by IEM (Fig. 4 E), and likely results from cleavage within the juxtamembrane region by lysosomal proteases. These data show that formation of intact fibrils correlates with appearance of TX-insoluble $M\alpha$.

The premelanosome fibrils contain TX-insoluble $M\alpha$

If the fibrils consist of $M\alpha$ fragments, they should label with antibodies to the Pmel17 luminal domain, but not to the cytoplasmic domain. IEM analyses confirm this prediction. In MNT-1 cells, α PEP13h labels the coated endosome precursor to stage II premelanosomes, including the ILVs within them, but not the striated stage II premelanosomes themselves (Fig. 6 A; also see supplemental materials in Raposo et al., 2001, available at <http://www.jcb.org/cgi/content/full/152/4/809/DC1/3>). Concordantly, in transfected HeLa cells expressing high levels of Pmel17, α PEP13h labeling is observed on the ILVs of MVBs (Fig. 6 B), but not on the striated regions that form within the same multivesicular structures (Fig. 6 C). By

Figure 6. Failure to detect Pmel17 COOH terminus in striated regions of premelanosomes and MVBs. Ultrathin cryosections of (A) MNT-1 or (B and C) transiently transfected HeLa cells expressing wild-type Pmel17 were double immunogold-labeled with HMB50 and PAG-10 and with α Pep13h and PAG-15. (A) A section of MNT-1 cells showing labeling by both antibodies on multivesicular coated endosomes (asterisk and inset), but only by HMB50 on stage II premelanosomes (II). Note the intraluminal labeling by α Pep13h in the marked coated endosome (asterisk). (B) A typical section of transfected HeLa cells containing abundant multivesicular compartments. Note the labeling with both antibodies, with HMB50 labeling on the luminal face and α PEP13h labeling primarily on the interior of the vesicles. (C) A section from a transfected HeLa cell demonstrating nascent striations. Note the absence of α PEP13h labeling in the striated regions (arrows) and on closely apposed membranes (arrows). Bars, 200 nm.



contrast, antibodies to the Pmel17 luminal domain label both the ILVs and the striations (Fig. 6, B and C). These data confirm that the fibrils lack the cytoplasmic domain present on full-length Pmel17 and the M β fragment.

To determine directly whether the premelanosome fibrils contain TX-insoluble M α fragments, MNT-1 cells were analyzed by subcellular fractionation. Light and dense fractions were harvested from post-nuclear cell homogenates by differential centrifugation, and membranes isolated from each fraction were subfractionated into TX-soluble and -insoluble pools (Fig. 7 A). The TX-soluble pools of both fractions contained P1 and Pmel17-s (representing pre-Golgi-localized Pmel17) and M β (Fig. 7 B, lanes 6 and 9); these products were nearly completely excluded from the TX-insoluble pool (lanes 7 and 10). By contrast, although M α was also distributed among both membrane fractions, within the dense membrane fraction it was enriched in the TX-insoluble pool (Fig. 7 B, lane 7). IEM analyses of the dense membrane fraction revealed melanosomes of all stages and other dense organelles such as lysosomes and late endosomes (Fig. 7, C and D). The limiting membranes of mature melanosomes labeled densely for Tyrp1 (Fig. 7 C); very little labeling for Pmel17 was observed on these nonpermeabilized, fixed samples (Fig. 7 D), likely due to sequestration within the lumen at a distance from the limiting membrane. As expected, no intact structures were recovered from the TX-soluble pool by IEM (unpublished data). By contrast, the insoluble pool consisted primarily of masses of pigment and nonpigmented fibrous structures, largely free of limiting membranes, that appeared to derive from stage II–IV melanosomes (Fig. 7, E–G). The fibers labeled densely with antibody to the Pmel17 luminal domain (Fig. 7, E and G), but not with α Pep13h (not depicted) or antibody to Tyrp1 (Fig. 7 F); by contrast, remaining limiting membrane surrounding the pigment labeled with antibody to Tyrp1 but not to Pmel17 (Fig. 7 F). Thus, TX-insoluble fibers contain TX-insoluble M α fragments. Together, the data show that formation of striations results at least in part from the incorporation of M α fragments into nonmembrane associated, TX-insoluble fibrous structures.

Discussion

Morphogenetic properties of resident intraorganellar proteins must be tightly regulated to ensure that organelle biogenesis proceeds at the proper place and time. One way to regulate these properties is by specific modifications of resident proteins during passage through transport intermediates. Our results demonstrate that premelanosome morphogenesis is regulated at the level of initiating intraorganellar fibrillogenesis through controlled PC-dependent proteolysis of a resident glycoprotein. These observations may reflect a more general requirement for regulated proteolysis of resident proteins by PCs and other proteases in the biogenesis of lysosome-related organelles; a similar model has been invoked for the formation of Weibel-Palade bodies in endothelial cells (Journet et al., 1993) and lamellar bodies in lung epithelial cells (Guttentag et al., 2003). Furthermore, our results extend the parallels between lysosome-related organelles and conventional dense core secretory granules, for

which a biogenetic role for PCs is well established (Arvan and Castle, 1998). Finally, our data elucidate the molecular basis for a well-known but enigmatic feature of melanosome morphology, the intraluminal fibrous striations, and show a striking parallel between the physiologic formation of these fibrils and the pathogenic formation of amyloid fibers.

Role of PCs in organelle morphogenesis

PC cleavage is required to activate numerous secreted and cell surface proproteins and viral fusion proteins (Thomas, 2002), but few intracellular substrates are known. Here, we used two approaches to show that cleavage of Pmel17 by furin or a related PC is required to generate premelanosome fibrils. The formation of both fibrils and the mature Pmel17-derived fragments, M α and M β , was blocked by (1) mutagenesis of the PC recognition site of Pmel17 in an exogenous expression system, and (2) inhibition of PC activity in a melanocytic cell line. Previous reports demonstrated defects in Weibel-Palade bodylike structures in an ectopic expression system upon mutagenesis of a PC site in von Willebrand factor (Journet et al., 1993), and in lamellar body formation in lung epithelial type II cells upon treatment with a cysteine protease inhibitor (Guttentag et al., 2003). However, this is the first time that both approaches have been used to conclusively demonstrate a role for PCs or other proteases in the activation of a single substrate protein to initiate morphogenesis of a lysosome-related organelle.

The relevant PC in melanocytic cells may be furin; furin is expressed in melanocytic cells (unpublished results), is capable of cleaving Pmel17 in reconstituted LoVo cells (Fig. 1 E), and is somewhat selectively inhibited by α_1 -PDX (Jean et al., 1998). On the other hand, the complement of PCs expressed by pigment cells is not known, and a different PC may function physiologically because α_1 -PDX can inhibit other PCs (Jean et al., 1998), and the Pmel17 cleavage site differs from the consensus furin recognition site (Fig. 1 B). We have shown that cleavage occurs within an acidic, post-Golgi, prelysosomal compartment that kinetically precedes stage II premelanosomes (Berson et al., 2001), consistent with the known localization of furin and other PCs to the TGN, endosomes, and secretory granules (Molloy et al., 1999; Thomas, 2002). The residual cleavage that occurs in the presence of brefeldin A and that results in a small pool of endoH-sensitive M β (Berson et al., 2001), like the residual cleavage of Pmel17 Δ cs in LoVo cells (Fig. 1 E), is likely mediated by NARC-1 or a related protease (Seidah et al., 2003). Of note, the accumulation of Golgi-processed Pmel17 observed upon inhibition of PC cleavage adds to the growing evidence that premelanosomes derive from post-Golgi compartments, and not from smooth ER (Kushimoto et al., 2001).

In MNT-1 cells expressing α_1 -PDX, striated structures were less abundant than in control cells, and many cells displayed numerous abnormal pigment-containing structures (Fig. 4). Because melanogenic enzymes such as tyrosinase and Tyrp1 are normally delivered to preformed stage II premelanosomes (Raposo et al., 2001), these unusual pigmented structures likely represent the products of malformed premelanosomes, depleted of fibrils, to which melanogenic enzymes were still delivered. The failure to completely deplete stage II

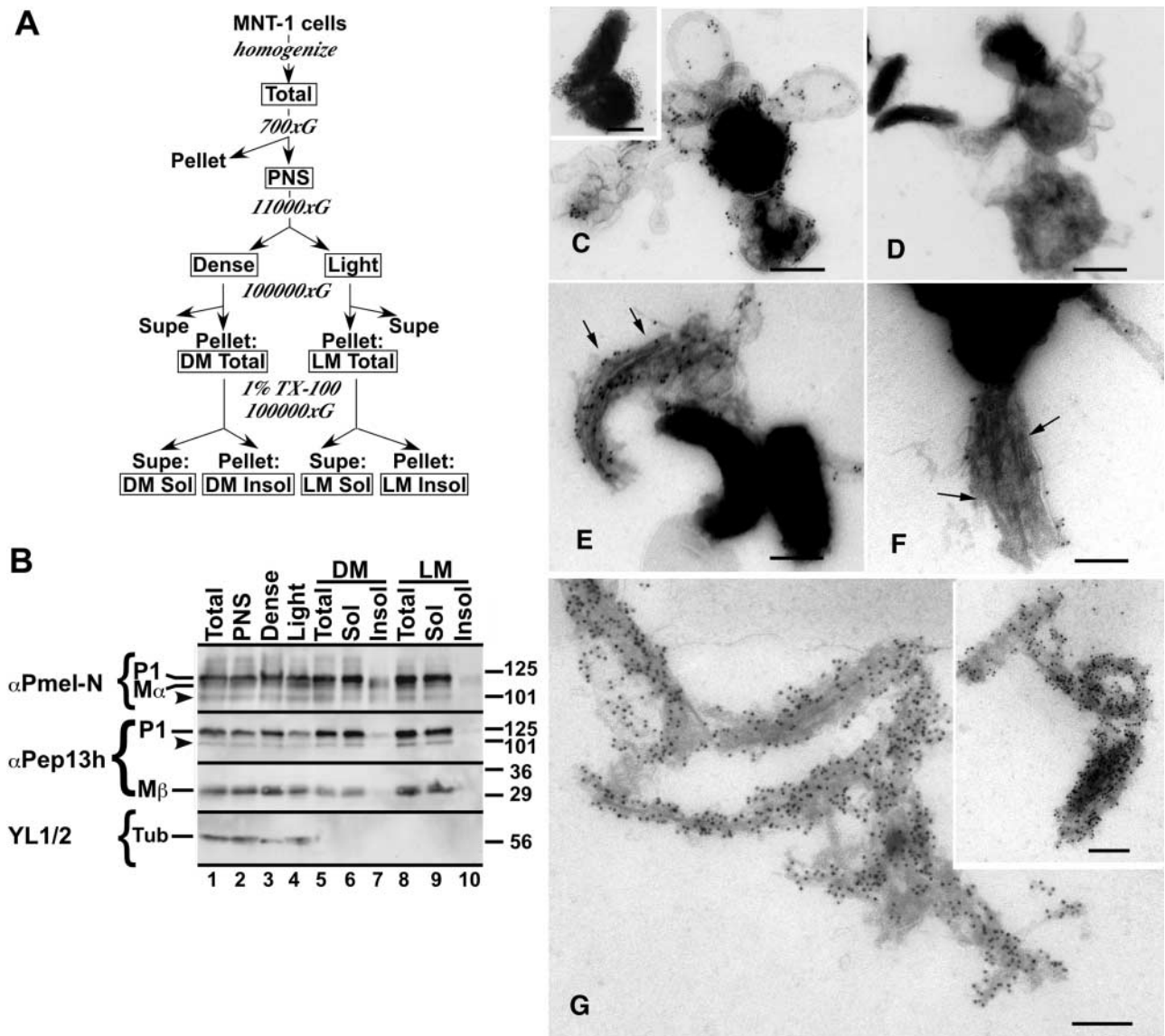


Figure 7. Cofractionation of TX-insoluble M α and premelanosome fibers. (A) Scheme for fractionation of post-nuclear supernatants (PNS) from homogenates of MNT-1 cells. Boxed fractions were analyzed by Western blotting in B. (B) Equal cell equivalents from each fraction were analyzed by SDS-PAGE and Western blotting with α Pmel-N, α Pep13h, or the anti-tubulin antibody YL 1/2, as indicated. Shown are the relevant portions of each gel; positions of molecular weight markers (right) and relevant proteins (left) are indicated. Note the enrichment of only M α in the TX-insoluble pool of the DM fraction (lane 7). (C–G) IEM analyses of relevant fractions using HMB45 (to Pmel17 luminal domain) or TA99 (to Tyrp1) and PAG-10. (C and D) Nonsolubilized membranes from the dense fraction (lane 5 in B) labeled with TA99 (C) or HMB45 (D). Note the labeling for Tyrp1 on the limiting membrane of highly pigmented structures (particularly in inset), and the absence of HMB45 labeling on these nonpermeabilized, whole-mounted fractions. (E–G) TX-insoluble membranes from the dense fraction (lane 7 in B) labeled with HMB45 (E and G) or TA99 (F). Note the dense labeling of fibrous structures (arrows), including relatively intact contents of a stage II premelanosome (G, inset), by HMB45. TA99 only sparsely labels residual limiting membranes (F, arrows). Bars, 200 nm.

premelanosomes in α_1 -PDX-treated cells was likely due to the short duration of expression after adenovirus infection; “normal” melanosomes of all stages present within α_1 -PDX-expressing cells likely represent later stage melanosomes that were in the process of forming, and/or partial fibril formation by a pool of properly cleaved Pmel17 that was present within abundant stage I premelanosomes, before a threshold of α_1 -PDX expression. Tonic exposure to α_1 -PDX for extended periods of time would likely further reduce the level of normal melanosomal structures in these cells, and may permit a timeline for melanosome maturation to be derived.

Composition and formation of striations and parallels with fibrillary diseases

The striated fibrils of stage II and III melanosomes have been thought to consist of protein with no underlying membrane based on their periodicity of electron lucence and density and the frequent appearance of a “zigzag” shape by EM (Birbeck et al., 1956; Moyer, 1966; Maul, 1969; Hearing et al., 1973). This interpretation seemed inconsistent with the ability of the integral membrane protein, Pmel17, to induce formation of and become incorporated into the striations (Berson et al., 2001; Raposo et al., 2001). We now reconcile these observa-

tions by showing that the PC cleavage product, M α , is eventually released from membranes and accumulates in TX-insoluble complexes. The insolubility of these complexes explains our previous inability to observe M α released from M β in the TX-soluble fraction (Berson et al., 2001) and the short half-life of TX-soluble Pmel17 (Kobayashi et al., 1994; Donatien and Orlow, 1995; Berson et al., 2001), and supports earlier evidence for Pmel17-derived fragments in detergent-insoluble fractions of partially purified melanosomes (Orlow et al., 1993; Zhou et al., 1994). The identity of M α is confirmed by (1) its immunoreactivity with α Pmel-N but not α Pep13h and its insensitivity to endoH; (2) the failure to generate M α and M β upon mutagenesis of KR⁴⁶⁹, inhibition of PCs by α_1 -PDX, or expression in PC-deficient LoVo cells; (3) the lack of any other potential dibasic cleavage sites in Pmel17 that could generate the appropriate sized fragments; and (4) the co-migration of intracellular M α with secreted Pmel17 (Berson et al., 2001), for which the sequence is known to end at V⁴⁶⁷ (Maresh et al., 1994). The presence of TX-insoluble, M β -dissociated M α on isolated fibrils and the dependence of striation formation on PC cleavage of Pmel17 indicates that (1) Pmel17 is not only sufficient, but also necessary for the generation of striations; and (2) the striations consist of insoluble complexes composed at least in part of M α without M β and underlying membrane.

How does M α become incorporated into detergent-insoluble complexes? M α must first be released from the integral membrane M β fragment, to which it is disulfide bonded on ILVs of multivesicular endosomes (Berson et al., 2001; Raposo and Marks, 2002). By analogy to the “sorting by retention” model of secretory granule formation (Arvan and Castle, 1998), the MVB may provide a structural framework to sequester Pmel17 and other molecules required for striation formation from other endosome components. Release of M α from the ILV must be mediated by either reduction of the disulfide bonds or proteolytic digestion of M β ; the latter is more likely, given the oxidative nature of melanin intermediates, the presence of lysosomal hydrolases in melanosomes (Orlow, 1995), and evidence for proteolytic maturation of Pmel17 in stage II premelanosome-enriched subcellular fractions (Chiamenti et al., 1996; Kushimoto et al., 2001). We speculate that release of M α from M β is accompanied by a conformational change that favors fibrillogenesis, perhaps facilitated by the low pH of forming premelanosomes (Raposo et al., 2001). Luminal segments within M β may serve to inhibit fibrillogenesis because longer Pmel17 fragments generated upon inhibition of PCs fail to properly form fibrils.

This model for physiological fibril formation in premelanosome biogenesis is reminiscent of the pathological process of fibril formation in a number of amyloid diseases. A specific requirement for PC cleavage and/or acidification in the formation of fibrils is observed in several pathogenic fibrillogenetic substrates, including gelsolin in familial amyloidosis of Finnish type (Chen et al., 2001), BRI-L in familial British dementia (Kim et al., 1999), and pro-islet amyloid polypeptide in type II diabetes (Badman et al., 1996). Proteolytic maturation, acidification, and/or association with detergent-insoluble lipid rafts—the lipid contents of which are similar to the ILVs of MVBs (Mobius et al., 2002; Wubbolts et al., 2003)—are common features in fibril formation by the amy-

loidogenic peptide in Alzheimer's disease (Esler and Wolfe, 2001; Ehehalt et al., 2003) and by prions (Taraboulos et al., 1995; Mayer et al., 1996; Zou and Cashman, 2002). Defining the fibrillogenetic determinant within the Pmel17 luminal domain and characterizing its properties will likely provide insights into the mechanism by which fibril formation is favored by proteolytic maturation, not only in premelanosomes, but also in these clinically important substrates.

Implications for biogenesis of lysosome-related organelles

PCs are well known to regulate the formation of dense cores in conventional secretory granules by permitting ordered aggregation and dense packing of granule contents (Arvan and Castle, 1998). Our work, together with the presumed role of PCs or other proteases in the formation of Weibel-Palade bodies or lamellar bodies, respectively, demonstrates that proteolytic activation plays a general role in lysosome-related organelle biogenesis as well. Other fibrillogenetic substrates may include Langerin, a C-type lectin that is involved in the formation of Birbeck granules in Langerhans cells (Valladeau et al., 2000). Future studies will likely confirm the generality of this biogenetic function of proteases, and may reveal whether fibrillogenetic activation steps are disrupted by genetic diseases of organelle biogenesis, such as Hermansky-Pudlak Syndrome (Spritz, 1999; Swank et al., 2000).

Materials and methods

Cell culture and transfections

MNT-1 human melanoma cells and furin-deficient LoVo adenocarcinoma cells (American Type Culture Collection) were maintained in DME containing 10% AIM-V medium (Life Technologies), 20% FBS, nonessential amino acids, sodium pyruvate, and antibiotics. HeLa cells were cultured as described previously (Berson et al., 2001). Cells were transiently transfected using either calcium phosphate precipitation (Marks et al., 1996) or FuGENE™ 6 (Roche) for HeLa or LipofectAMINE™ (Life Technologies) for LoVo according to manufacturer's instructions. Cells were analyzed 2–3 d after transfection.

Antibodies

mAb specific for Tyrp1 (TA99/Mel-5; Thomson et al., 1985) and affinity-purified rabbit antibody to the Pmel17 COOH terminus (α PEP13h; Berson et al., 2001) were described previously. Purchased mAbs were HMB50 and HMB45 (anti-Pmel17; Lab Vision), M2 (anti-FLAG; Sigma-Aldrich), H4A3 (anti-LAMP1; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-CD63 (Immunotech), and YL 1/2 (anti-tubulin; Accurate Chemical). Rabbit antiserum α Pmel-N was generated by Genemed Synthesis, Inc. to a synthetic peptide (CTKVPRNQDWLGVSRQLR-CO₂H) corresponding to the human Pmel17 NH₂ terminus (residues 24–40), and affinity purified on peptide coupled to SulfoLink beads (Pierce Chemical Co.). α Pmel-N had no reactivity with untransfected HeLa cells. Protein A gold conjugates were purchased from Dr. J. Slot (Utrecht Medical School, Utrecht, Netherlands).

Plasmids

pCI-Pmel17 encoding the Pmel17 long form in pCI (Promega) has been described previously (Berson et al., 2001). Pmel17 Δ cs was generated by site-directed mutagenesis using two-step amplification (Higuchi et al., 1988), replacing the endogenous sequence using unique KasI and NsiI sites, and sequence verified by automated dideoxy sequencing. HA11 epitope-tagged furin in pSX (Bosshart et al., 1994) was a gift of Dr. J.S. Bonifacino (National Institutes of Health, Bethesda, MD).

Recombinant virus production and infection

Recombinant adenoviruses encoding the tetracycline-responsive transactivator (tTA) and tetracycline-responsive, FLAG-epitope tagged α_1 -PDX and α_1 -AT were gifts of Drs. G. Thomas and L. Thomas (Vollum Institute, Ore-

gon Health Sciences University, Portland, OR; Jean et al., 2000). Viruses were amplified and titred using the Tissue Culture Infectious Dose 50 method according to the AdEasy™ vector system application manual (Qbiogene). Crude virus preparations in PBS were used directly for double infections of MNT-1 cells, according to the AdEasy™ manual, using tTA virus at moi of 20 and α_1 -PDX or α_1 -AT virus at moi of 5 or 20. Infections were not cytotoxic for MNT-1 cells as judged by trypan blue exclusion, and resulted in transgene expression in $\geq 50\%$ of the cells by IFM. Cells were harvested 2 d after infection for analyses.

Metabolic labeling and immunoprecipitation

Cells were metabolically pulse labeled for 15–30 min with [³⁵S]methionine/cysteine and chased for indicated times as described previously (Marks et al., 1996). Fresh or frozen cell pellets were lysed in 1% (wt/vol) TX for 30 min as described previously (Berson et al., 2000), and lysates were clarified by centrifugation for 15 min at 20,000 g. Where noted, the resulting TX-insoluble pellets were resuspended in 50 μ l TX lysis buffer containing 8M urea and heated at 60°C for 10 min. After cooling, samples were diluted 20 \times with TX lysis buffer for immunoprecipitation. Immunoprecipitations, SDS-PAGE on 10% polyacrylamide gels, and phosphorimaging analysis were performed as described previously (Berson et al., 2000). For sequential immunoprecipitations, unbound material from the first precipitation was clarified by centrifugation at 20,000 g for 5 min before incubation with the subsequent immobilized antibody.

Immunoblotting

Western blotting using whole-cell lysates prepared with 1% SDS, and endoH/ N-glycanase F treatments were as described previously (Berson et al., 2000). For fractionation into TX-soluble and -insoluble fractions, 10⁶ cells were resuspended in 200 μ l lysis buffer containing 1% TX and protease inhibitors at 4°C for 2 h. After centrifugation at 20,000 g for 15 min, supernatants (TX soluble) were harvested and 6 \times SDS-PAGE sample buffer was added before analysis. Pellets (TX insoluble) were washed once with 1% TX lysis buffer and then resuspended in 1 \times SDS-PAGE sample buffer for analysis. 8% PAGE/5% methanol transfer buffer was used for α Pmel-N and antitubulin blots, and 12% PAGE/15% methanol transfer buffer for α Pep13h blots. Immobilon-P membranes (Millipore) with transferred proteins were probed with indicated antibodies, and bands were detected with alkaline phosphatase-conjugated goat anti-rabbit Ig, enhanced chemiluminescence, and phosphorimaging analysis using a fluorescence imaging system (Storm® 860; Molecular Dynamics, Inc.) and ImageQUANT software (Amersham Biosciences).

Subcellular fractionation

See Fig. 7 A for schematic. MNT-1 cells ($\sim 10^8$) were homogenized with a hand-held Dounce homogenizer in 4 volumes of buffer I (25 mM Hepes, 1 mM EDTA, 0.1 mM EGTA, and 0.02% sodium azide, pH 7.4) containing 0.25 M sucrose and a cocktail of protease inhibitors (Berson et al., 2000). Cell debris and nuclei were pelleted at 700 g for 10 min. Post-nuclear supernatants were layered over a cushion of 2.0 M sucrose in buffer I and centrifuged at 11,000 g for 10 min. The black interface at the cushion (dense membrane fraction) and the diffuse supernatant (light membrane fraction) were harvested, and each was subjected to 100,000 g for 60 min to recover a pellet (total membrane fraction). Pellets were resuspended in 600 μ l 150 mM NaCl/0.1 M Tris-HCl, pH 8.0; after a sample was removed for EM analysis, TX was added to a final concentration of 1%, and samples were rotated at 4°C for 2 h. Samples were then spun at 20,000 g for 15 min, and supernatants (TX-soluble fraction) and pellets (TX insoluble fraction) were harvested. Approximately 3% of the material in each fraction was used for each lane for Western blotting analyses. An aliquot of each fraction was fixed for EM analysis as described below.

Electron microscopy

Conventional EM of MNT-1 or transfected HeLa cells was performed as described previously (Raposo et al., 2001). For IEM, cells were fixed with 2% PFA/0.2% glutaraldehyde and single- or double-immunogold labeling of ultrathin cryosections was performed as described previously (Raposo et al., 1997, 2001) using protein A conjugated to 10-nm or 15-nm gold particles (PAG-10 or -15). For transfected HeLa cells, only cells expressing comparable levels of wild-type Pmel17 and Pmel17 Δ c were analyzed in parallel. Expression per cell was assessed as total expression level (judged by Western blotting) divided by percentage of transfected cells (judged by IFM). Relative quantitation as described in the text was performed directly under the electron microscope by counting the number of affected and unaffected relevant compartments among 50 cell profiles. For EM analysis of subcellular fractions, 20- μ l drops of each fraction, previously fixed with

2% PFA in PHEM buffer, as described in Raposo et al. (2001), were placed on formvar-carbon-coated EM grids for 30 min. The grids were then immunogold labeled, contrasted, and embedded in a mixture of uranyl acetate and methylcellulose as described for ultrathin cryosections (Raposo et al., 1997).

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