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

Joanne F. Berson,1 Alexander C. Theos,1 Dawn C. Harper,1 Danielle Tenza,2 Graça Raposo,2 and Michael S. Marks1

1Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104
2Institut Curie, Centre National de la Recherche Scientifique UMR 144, 75005 Paris, France

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 melanosomal 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; disregulation may result in disease, as exemplified by the defects in lysosome-related organelles in patients 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
Step in melanosomal 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 premelanosomal matrix (Kwon et al., 1987; Venegoor 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 melanosomal function is supported by the pigmentation defect in silver mice (Dunn and Thigpen, 1930), in which the Pmel17 protein is truncated (Martinez-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 premelanosomal-like fibrils within multivesicular bodies (MVBs)* of nonpigment cells (Berson et al., 2001). Nevertheless, Pmel17 in nonpigmenting 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 nonmembranous 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 Mβ fragment containing part of the luminal domain and the entire transmembrane and cytoplasmic domains, and an ~80-kD Mα 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 Mα, free of Mβ, is precipitated from cell supernatants (Berson et al., 2001). Mα migrates identically to ME-20s, consisting of

was enhanced fourfold to emphasize the failure to detect Mβ from Pmel17acs. Migration of molecular weight markers and P1, P2, Mα, and Mβ forms of Pmel17 are indicated. (D and E) Western blot analysis of wild-type Pmel17 (WT) and Pmel17acs (acs) 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 αPep13h with enhanced chemiluminescence. Positions of molecular weight markers and P1 and Mβ forms of Pmel17 are indicated.

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 lumenal region deleted in the alternative spliced form, Pmel17-s, are indicated; N-linked glycosylations are represented by branched structures; regions corresponding to Mα and Mβ are shown above; and regions recognized by antibodies αPmel-N, HMB50, and αPep13h are shown below. (B) Sequence of the conserved dibasic consensus site from orthologues human Pmel17 (GenBank/EMBL/DDJB accession no. NP_008859), chicken MMP115 (GenBank/EMBL/DDJB accession no. Q98917), bovine RPE1 (GenBank/EMBL/DDJB accession no. Q98917), equine Pmel17 (GenBank/EMBL/DDJB accession no. AAC97108), and mouse Silver (GenBank/EMBL/DDJB accession no. NP_068682). Amino acid positions are noted (equine and bovine sequences are from incomplete clones); conserved residues are in gray. Top, Pmel17acs (acs) 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 Pmel17acs (acs) were metabolically pulse labeled and chased as indicated. TX cell lysates at each time point were immunoprecipitated with αPep13h, fractionated by SDS-PAGE, and analyzed by phosphorimaging. Shown are regions of the gels encompassing the relevant bands; the signal from the lower region

was enhanced fourfold to emphasize the failure to detect Mβ from Pmel17acs. Migration of molecular weight markers and P1, P2, Mα, and Mβ forms of Pmel17 are indicated. (D and E) Western blot analysis of wild-type Pmel17 (WT) and Pmel17acs (acs) 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 αPep13h with enhanced chemiluminescence. Positions of molecular weight markers and P1 and Mβ forms of Pmel17 are indicated.
Pmel17 residues 25–467, identified in melanoma cell supernatants (Maresch 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/Mα 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→QQ by site-directed mutagenesis, generated Pmel17Δcs expressed in transfected HeLa cells was not processed to Mα and Mβ (Fig. 1, C and D). Instead, cells accumulated the P2 form, representing full-length Pmel17Δcs with Golgi-modified N-linked glycans (Berson et al., 2001). Furthermore, although cells expressing wild-type Pmel17 secrete low levels of Mα into the medium, cells expressing Pmel17Δcs did not (unpublished data).

To determine whether PCs are required for the cleavage, Pmel17 or the Δcs 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 αPEP13h, an antibody to the cytoplasmic domain. Lysates from HeLa cells expressing wild-type Pmel17, like melanocytic cells, contain two immunoreactive bands with M, 28,000 and ~100,000, representing Mβ and the full-length, endoglycosidase H (endo-H)-sensitive P1 precursor, respectively (Fig. 1 D; see Fig. 5 A). By contrast, in both HeLa cells expressing Pmel17Δcs (Fig. 1 D) and LoVo cells expressing wild-type Pmel17 (Fig. 1 E), only the ~100-kD P1 band and not Mβ was observed, indicating that cleavage did not occur (a faint residual band that co-migrates with Mβ in LoVo cells expressing Pmel17Δcs 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 KR68→QQ mutation; Seidah et al., 2003). Mβ was restored in LoVo cells by coexpression of both Pmel17 and furin, but not Pmel17Δcs and furin (Fig. 1 E). Thus, furin or a similar PC can cleave Pmel17 at the dibasic recognition site. Importantly, cleavage at the other dibasic sites in Pmel17, KR147, or RR192, would not generate fragments of the appropriate size for Mα and Mβ.

To determine whether a furin-like PC cleaves Pmel17 in melanocytic cells, we inhibited PCs in the pigmented human melanoma, MNT-1, by expressing α1-antitrypsin Portland (α1-PDX), a PC-specific variant of α1-antitrypsin inhibitor (α1-AT; Anderson et al., 1993; Jean et al., 1998, 2000). FLAG epitope–tagged α1-PDX or α1-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 multiplicity of infection (moi) with the α1-PDX virus was required to obtain the same level of expression as α1-AT (Fig. 2 A; likely due to contaminating nonrecombinants in the α1-AT preparation). As described previously, Pmel17 in uninfected cells first appears as endoH-sensitive P1 and Pmel17Δcs forms (Berson et al., 2001). P1 is the full-length core glycosylated precursor, and Pmel17Δcs 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); Pmel17Δcs is distinguishable from Mα based on its direct reactivity with αPep13h, its appearance in the pulse-labeled samples, and its sensitivity to EndoH (see Fig. 5). P1 and Pmel17Δcs are subsequently modified in the Golgi to endoH-resistant P2, and P2 is then cleaved to Mα and Mβ (Fig. 2 B, lanes 1–5). All fragments disappeared from detergent cell lysates with a half-life of 2.5 h. In cells expressing α1-AT, the pattern and kinetics of Pmel17 processing were virtually unchanged (Fig. 2 B, lanes 6–10). However, in cells expressing α1-PDX, as in HeLa cells expressing Pmel17Δcs, P2 accumulated, and generation of Mα/Mβ 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 α1-PDX (Fig. 2, B and C), likely reflecting the secretion of α1-PDX by infected cells and uptake by uninfected cells. By contrast, α1-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 intralumenal 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 Pmel17Δcs were analyzed by immunofluorescence microscopy (IFM) and immunoelectron microscopy (IEM). Pmel17Δcs, 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 Pmel17Δcs 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 α1-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...
pressing striation formation in melanocytic cells, MNT-1 cells express intermediates in the degradation of Pmel17 by lysosomes. The dense aggregates observed by IEM may represent concomitant with enhanced movement from late endosomes by PCs, its ability to nucleate striation formation is inhibited by 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 α₁-AT (moi of 20; A20) or α₁-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 α₁-AT and α₁-PDX immunoprecipitated from pulse-labeled cell lysates using an anti-FLAG antibody. Similar levels of transgene expression were obtained using α₁-PDX at moi of 5 and α₁-AT at moi of 20. (B) Pmel17 immunoprecipitated using aPEP13h. 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× to emphasize the Mβ band. Positions of molecular weight markers (right) and of P1, Pmel17-s, P2, Mα, and Mβ (left) are noted. Pmel17-s is the product of an alternatively spliced Pmel17 mRNA. (C) Quantitation of the signal intensity of P1, P2, and Mβ 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 Mβ represents 42% of that present in full-length Pmel17.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uninfected</th>
<th>A20</th>
<th>P20</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chase (h)</td>
<td>0 1 2 4 6</td>
<td>0 1 2 4 6</td>
<td>0 1 2 4 6</td>
<td>0 1 2 4 6</td>
</tr>
<tr>
<td>P2</td>
<td>29.5</td>
<td>97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HeLa cells expressing Pmel17 WT or Pmel17Δcs 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 Δcs).

These correspond to MVBs with non-organized proteinaceous material. Because α₁-PDX or α₁-AT are secreted, even uninfected cells in the cultures were partially subject to their effects. Although expression of α₁-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 α₁-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 Pmel17Δcs-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 intralumenal membranes of coated endosomes in MNT-1 cells was not affected by α₁-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 Mα fragments...
Figure 3. **Cleavage site-deficient Pmel17 localizes to MVBs, but fails to form fibers.** Transiently transfected HeLa cells expressing Pmel17Δcs (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 Pmel17Δcs-expressing cell showing predominant labeling on ILVs of multivesicular late endosomes. (B) A cell with very high Pmel17Δcs 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 Pmel17Δcs. Note the accumulation of electron-dense material in MVBs, but no obvious fibers (arrowheads). Bars, 200 nm.
Figure 4. Disruption of melanosomal morphology in MNT-1 cells expressing α₁-PDX. MNT-1 cells infected with recombinant adenoviruses expressing tTA and either α₁-AT (A and B) or α₁-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 α₁-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 α₁-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.
form intraluminal proteinaceous fibrils upon their release from membranes. By analogy to amyloid, such fibrils might be insoluble in non-denaturing detergents such as TX. Indeed, M\(\alpha\) was observed in TX-insoluble fractions of MNT-1 cells. By Western blotting, an endoH-resistant, N-glycanase F-sensitive M\(\alpha\) band was detected in whole MNT-1 cell lysates with \(\alpha\)Pep13h (antibody to the lumenally exposed NH\(_2\) terminus of Pmel17), but not with \(\alpha\)Pep13h (antibody to the cytoplasmically exposed COOH terminus; Fig. 5 A), distinguishing it from the endoH-sensitive, \(\alpha\)Pep13h-reactive alternative splice product, Pmel17-s. Although most M\(\beta\), Pmel17-s, and P1 isoforms were soluble in TX, a significant fraction of M\(\alpha\) was detected in the TX-insoluble pool (Fig. 5 B). Formation of TX-insoluble M\(\alpha\) was kinetically linked to both cleavage and dissociation from M\(\beta\) 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 αPep13h and then αPmel-N, then analyzed by SDS-PAGE and phosphorimaging. From the soluble pool, αPmel-N only immunoprecipitated residual P1, P2, Pmel17-s, Mα, and Mβ in similar proportions as in the initial αPep13h immunoprecipitates (Fig. 5, C and D; compare lanes 1–4 with lanes 5–8). From the TX-insoluble pool, although αPep13h immunoprecipitated only full-length P1 and Pmel17-s (Fig. 5 C, lanes 9–12), subsequent αPmel-N immunoprecipitates additionally contained an ~80-kD band (lanes 13–16); its failure to be depleted by αPep13h suggests that this band is Mα. 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 Mα was released from Mβ during the chase and accumulated in TX-insoluble complexes.

To confirm the identity of this TX-insoluble band as Mα and to determine whether its accumulation required PC cleavage, MNT-1 cells expressing α1-PDX were similarly analyzed by pulse/chase and immunoprecipitation. As shown in Fig. 5 F, expression of α1-PDX (but not α1-AT) inhibited the formation of TX insoluble Mα. Appearance of the fragment was both delayed and reduced in efficiency by expression of α1-PDX. Instead, these cells accumulated a larger fragment of Pmel17, lacking the COOH terminus and possibly comprising the entire lumenal 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 Mα.

The premelanosome fibrils contain TX-insoluble Mα

If the fibrils consist of Mα fragments, they should label with antibodies to the Pmel17 lumenal domain, but not to the cytoplasmic domain. IEM analyses confirm this prediction. In MNT-1 cells, αPEP13h 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, αPEP13h 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 αPep13h 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 intralumenal labeling by αPep13h 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 αPEP13h labeling primarily on the interior of the vesicles. (C) A section from a transfected HeLa cell demonstrating nascent striations. Note the absence of αPEP13h labeling in the striated regions (arrows) and on closely apposed membranes (arrows). Bars, 200 nm.
contrast, antibodies to the Pmel17 lumenal 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 Mβ fragment.

To determine directly whether the premelanosome fibrils contain TX-insoluble Mα 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 Mβ (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 Mα 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 αPep13h (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 Mα fragments. Together, the data show that formation of striations results at least in part from the incorporation of Mα 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, Mα and Mβ, 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 α1-PDX (Jeen 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 α1-PDX can inhibit other PCs (Jeen 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 secretary 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 Mβ (Berson et al., 2001), like the residual cleavage of Pmel17Δcs 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 α1-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
premelanosomes in α₁-PDX–treated cells was likely due to the short duration of expression after adenovirus infection; “normal” melanosomes of all stages present within α₁-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 α₁-PDX expression. Tonic exposure to α₁-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, Mα, is eventually released from membranes and accumulates in TX-insoluble complexes. The insolubility of these complexes explains our previous inability to observe Mα released from MB 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 Mα is confirmed by (1) its immunoreactivity with αPmel-N but not αPep13h and its insensitivity to endoH; (2) the failure to generate Mα and MB upon mutagenesis of KR469—an inhibition of PCs by α1-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 Mα with secreted Pmel17 (Berson et al., 2001), for which the sequence is known to end at V469 (Maresh et al., 1994). The presence of TX-insoluble, MB-dissociated Mα 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 Mα without MB and underlying membrane.

How does Mα become incorporated into detergent-insoluble complexes? Mα must first be released from the integral membrane MB 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 Mα from the ILV must be mediated by either reduction of the disulfide bonds or proteolytic digestion of MB; 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 Mα from MB is accompanied by a conformational change that favors fibrillogenic activity, perhaps facilitated by the low pH of forming premelanosomes (Raposo et al., 2001). Luminal segments within MB 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 fibrillogenic substrates, including gelsolin in familial amyloidoidoses 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; Wubbelts et al., 2003)—are common features in fibril formation by the amyloidogenic 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 fibrillogenic determinant within the Pmel17 lumenal 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 fibrillogenic 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 biogenic function of proteases, and may reveal whether fibrillogenic 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-3; Thomson et al., 1985) and affinity-purified rabbit antibody to the Pmel17 COOH terminus (αPEP13h; 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 antisera αPmel-N was generated by Genemed Synthesis, Inc. to a synthetic peptide (CTKVPRNQDWLGVSRQLR-COOH) corresponding to the human Pmel17 NH2 terminus (residues 24–40), and affinity purified on peptide coupled to Stul/O Link beads (Pierce Chemical Co.). αPmel-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). Pmel17Δcs was generated by site-directed mutagenesis using two-step amplification (Higuchi et al., 1988), replacing the endogenous sequence using unique Ksl and Nsil 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. Bonifácio (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 α1-PDX and α1-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 titrated 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 TIA virus at moi of 20 and α1-PDX or α1-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 ≥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 μl TX lysis buffer containing 8M urea and heated at 60°C for 10 min. After cooling, samples were diluted 20× 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 μl 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× SDS-PAGE sample buffer was added before analysis. Pellets (TX insoluble) were washed once with 1% TX lysis buffer and then resuspended in 1× SDS-PAGE sample buffer for analysis. 8% PAGE/5% methanol transfer buffer was used for αPmel-N and anti-tubulin blots, and 12% PAGE/15% methanol transfer buffer for αPep13h blots. Immobilon-P membranes (Millipore) with transferred proteins were probed with indicated antibodies, and bands were detected with alkaline phosphatase-conjugated goat anti-rabbit Igg, 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 (∼106) 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 for 1 hr at 100,000g. The TX-soluble fraction was fixed for EM analysis as described below. Each fraction was subjected to 100,000 g for 15 min at 20,000 g, and supernatants (TX soluble) and pellets (TX insoluble fraction) were harvested, and each was subjected to 100,000 g for 15 min at 20,000 g.

Electron microscopy

Conventional EM of MNT-1 or transfected HeLa cells was performed as described previously (Raposo et al., 2000). For EM analysis, cells were 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).

We thank G. Thomas and L. Thomas for gifts of α1-PDX, α1-AT, and tTA encoding adenoviruses, P. Arvan for suggesting their use, and P. Bates for technical help with adenovirus protocols. We also thank N. Seidah for helpful discussions and G. Thomas and E. Dell’Angelica for critical review of the manuscript.

This work was supported by National Institutes of Health grants R01 EY 22207 and R01 AR 48155 (to M.S. Marks) and by Association de Recherche pour le Cancer and Vaincre les Maladies Lysosomales (to G. Raposo). J.F. Berson was supported in part by training grant T32 CA 09140 from the National Cancer Institute and by American Cancer Society Fellowship PF-99-336-01-CIM.

References

Guttenbach, S., L. Robinson, P. Zhang, F. Brasch, F. Buhlig, and M. Beers. 2003. Western blotting was performed as described previously (Raposo et al., 1997).

We thank G. Thomas and L. Thomas for gifts of α1-PDX, α1-AT, and tTA encoding adenoviruses, P. Arvan for suggesting their use, and P. Bates for technical help with adenovirus protocols. We also thank N. Seidah for helpful discussions and G. Thomas and E. Dell’Angelica for critical review of the manuscript.

This work was supported by National Institutes of Health grants R01 EY 22207 and R01 AR 48155 (to M.S. Marks) and by Association de Recherche pour le Cancer and Vaincre les Maladies Lysosomales (to G. Raposo). J.F. Berson was supported in part by training grant T32 CA 09140 from the National Cancer Institute and by American Cancer Society Fellowship PF-99-336-01-CIM.

Submitted: 12 February 2003
Revised: 18 March 2003
Accepted: 19 March 2003
Proprotein convertases and melanosome biogenesis | Berson et al. 533


