Premelanosomal Amyloid-like Fibrils Are Composed of Only Golgi-processed Forms of Pmel17 That Have Been Proteolytically Processed in Endosomes*

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Melanin pigments are synthesized within specialized organelles called melanosomes and polymerize on intraluminal fibrils that form within melanosomal precursors. The fibrils consist of proteolytic fragments derived from Pmel17, a pigment cell-specific integral membrane protein. The intracellular pathways by which Pmel17 accesses melanosomal precursors and the identity of the Pmel17 derivatives within fibrillar melanosomes have been a matter of debate. We show here that antibodies that detect Pmel17 within fibrillar melanosomes recognize only the luminal products of proprotein convertase cleavage and not the remaining products linked to the transmembrane domain. Moreover, antibodies to the N and C termini detect only Pmel17 isoforms present in early biosynthetic compartments, which constitute a large fraction of detectable steady state Pmel17 in cell lysates because of slow early biosynthetic transport and rapid consumption by fibril formation. Using an antibody to a luminal epitope that is destroyed upon modification by O-linked oligosaccharides, we show that all post-endoplasmic reticulum Pmel17 isoforms are modified by Golgi-associated oligosaccharide transferases, and that only processed forms contribute to melanosome biogenesis. These data indicate that Pmel17 follows a single biosynthetic route from the endoplasmic reticulum through the Golgi complex and endosomes to melanosomes, and that only fragments encompassing previously described functional luminal determinants are present within the fibrils. These data have important implications for the site and mechanism of fibril formation.

Melanin pigments function in photoprotection in the skin and ocular development and visual acuity in the eye. They are synthesized and stored within specialized lysosome-related organelles of melanocytes and ocular pigment epithelia called melanosomes (1, 2). Melanosomes bearing brown and black melanins, or eumelansins, develop within melanosocytes through four morphologically distinct stages. Stage I and II melanosomes lack melanins and are characterized by the progressive development of intraluminal fibrillar striations, upon which melanins are deposited as they are synthesized in stages III and IV. The fibrils likely serve to detoxify oxidative melanin intermediates and concentrate them for storage (in ocular pigment cells) or for transfer to keratinocytes (in epidermal melanocytes) (3). The fibrils resemble amyloid both morphologically and structurally (4). Thus, understanding the nature of their formation may help to decipher mechanisms controlling pathological amyloid biogenesis.

The major biogenetic component of the melanosome fibrils is the pigment cell-specific protein, Pmel17 (also known as gp100 or SILV; referred to here as Pmel).3 Pmel is the only pigment cell-specific protein required for fibril formation, as its ectopic expression in non-pigment cells is sufficient to induce the formation of melanosome-like fibrils (5). Conversely, Pmel gene mutations are associated with hypopigmentation in several animal models (6–11), including silver mice (9) in which eumelanosomes are depleted of fibrils and altered in morphology (12). Pmel immunoreactivity is detected on fibrils in stage II melanosomes (13, 14), and Pmel fragments copurify with fibrils (15) or stage II melanosomes (16, 17) by subcellular fractionation. Finally, purified recombinant Pmel fragments produced in bacteria adopt a fibrillar conformation in vitro (4), suggesting that Pmel may be the sole component of the melanosome fibrils.

Although Pmel is clearly a critical component of melanosome fibrils, the mechanism by which Pmel adopts a fibrillar conformation in vivo remains unknown. To define this mechanism, it is critical to clearly understand Pmel biosynthetic trafficking within melanosomes to compartments in which fibrils form. Human Pmel is synthesized as a type I integral membrane protein with an N-terminal signal sequence, a large luminal domain, a single 24-residue membrane-spanning domain, and

3 The abbreviations used are: Pmel, Pmel17; CS, proprotein convertase cleavage site; EndoH, endoglycosidase H; ER, endoplasmic reticulum; IFM, immunofluorescence microscopy; KLD, Kring-like domain; NTR, N-terminal region; PE, phycoerythrin; PKD, polycystic kidney disease-1 repeat-like region; PNGase F, protein N-glycanase F; RPT, region of internal repeats; WT, wild-type; CHO, CHO-K1 cells; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ERGIC, ER-Golgi intermediate compartment.
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a 45-residue cytoplasmic domain (18–20). Four Pmel products with luminal domains of 525, 532, 567, and 574 residues result from alternatively spliced mRNAs (21–23), with the 567-residue form predominating in most melanocytic cells. As for other type I integral membrane proteins, the signal sequence is cleaved, and at least four core-N-linked oligosaccharides are added to consensus attachment sites, presumably cotranslationally, in the endoplasmic reticulum (ER) (5, 24). At least a fraction of Pmel traverses the Golgi complex, where some of the N-linked oligosaccharides are modified by resident mannosidases and glycosyltransferases to a complex form that is resistant to digestion in vitro by endoglycosidase H (EndoH) (5, 24) and where O-linked oligosaccharides are added, elaborated, and modified by sialic acid (25). At least a fraction of the mature form is cleaved into two fragments, referred to here as $\alpha$ and $\beta$, in a post-Golgi compartment (5) (likely endosomes; see Ref. 26) by a proprotein convertase (15). A small fraction of the resulting luminal fragment is secreted (5, 24). Pmel that accumulates in fibrillar stage II melanosomes is reactive with three commonly used antibodies, HMB45, HMB50, and NKI-beteb, both well established to identify stage II melanomas (16, 17). Epitope mapping studies by Yasumoto et al. (28) assigned antibody reactivity to monoclonal antibodies HMB50 and NKI-beteb, both well established to identify stage II melanomas by immunoelectron microscopy (14, 15, 36), to membrane-proximal regions of the luminal domain that should be absent in $\alpha$. A recent study showed that Pmel is modified by O-linked oligosaccharides, a modification that occurs in the Golgi complex (37), but the results were interpreted to conclude that an alternative post-ER form of Pmel lacking O-linked oligosaccharides is found in melanomas (25). Because immature forms of Pmel also copurify by subcellular fractionation with clathrin-associated adaptor complexes AP-1 and AP-2 (38), which are known to facilitate endosomal protein sorting from the trans-Golgi network, endosomes, and the plasma membrane (39), it was concluded that Pmel reaches endocytic compartments without passing through the Golgi complex (25, 38). These conclusions were supported by immunofluorescence microscopy (IFM) analyses in which labeling by antibodies that react with immature forms of Pmel, but not with $\alpha$, overlapped with labeling by HMB45/HMB50/NKI-beteb and by adaptor complexes, respectively (16, 25, 28, 38).

Many of the data supporting the second model are based on subcellular fractionation, the results of which might reflect impurities in isolated fractions. Several conclusions, however, seem incompatible with the first model, including the following: (i) the epitope reactivity of several of the antibodies; (ii) the inferred detection of Pmel isoforms with stage II melanomas by IFM. Here we present data that counter these conclusions and suggest that the data supporting them might warrant reevaluation. We show that immature forms of Pmel are found only in pre-Golgi compartments of melanocytic cells and that stage II melanosomes harbor only Golgi-modified Pmel fragments that are derived from $\alpha$ and that bear sialylated O-linked oligosaccharides. The results do not support a model in which a distinct cohort of Pmel accesses melanosomes either directly from the ER or with unmodified oligosaccharides. Nevertheless, we show that neither N- nor O-linked glycans are required for trafficking of Pmel through the conventional biosynthetic pathway to melanosome precursor compartments, and we discuss the potential roles of different types of glycosylation in fibrillogenesis.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—All chemicals were obtained from Sigma or Thermo Fisher Scientific (Fremont, CA) unless stated otherwise. The following mAbs were used: HMB45,
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HMB50, and NKI-beteb to human Pmel were purchased from LabVision/Thermo Fisher Scientific (Fremont, CA); XD5.A11 to human leukocyte antigen class II β chains (40) and TA99 (also called mel-5) anti-Tyrp1 (41) were produced in-house from hybridomas originally purchased from American Type Culture Collection (Manassas, VA); mAb G1/93 to ERGIC-53 (42) was from Axxora (San Diego) or a kind gift of H. P. Hauri (University of Basel, Basel, Switzerland); mAb 3126 to calnexin was from Chemicon (Temecula, CA); and anti-tubulin was from Sigma. The following polyclonal anti-peptide antibodies were prepared in-house and affinity-purified as described previously: αPep13h to the C-terminal 14 residues of human Pmel (14) (identical to αPep13h generated by Kushimoto et al. (16)); αPmel-N to the N-terminal 17 residues of human Pmel (15, 23); and αPmel-l to residues 326–344 of human Pmel (23). Rabbit anti-Tac to human interleukin-2 receptor α chain (43) or pre-immune serum was used as a negative control. Rabbit anti-LAMP-1 was purchased from Affinity BioReagents (Golden, CO).

Cell Culture and Transfections—MNT-1 human melanoma cells (44) and HeLa cells were cultured as described previously (5, 14). CHO-K1 and ldlD14 cells (45) were obtained with the kind permission of Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge) from American Type Culture Collection and were cultured in Ham’s F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen). HeLa cells were subjected to secondary immunoprecipitations exactly as described previously: αPep13h to the C-terminal 14 residues of human Pmel (14) (identical to αPep13h generated by Kushimoto et al. (16)); αPmel-N to the N-terminal 17 residues of human Pmel (15, 23); and αPmel-l to residues 326–344 of human Pmel (23). Rabbit anti-Tac to human interleukin-2 receptor α chain (43) or pre-immune serum was used as a negative control. Rabbit anti-LAMP-1 was purchased from Affinity BioReagents (Golden, CO).

Expression Plasmids—Human Pmel (long form, hPmel17-l) and Pmel-s (human Pmel17-s or short form) in the pCI vector (Promega, Madison, WI) have been described (23). Deletion mutants ΔNTR, ΔPKD, ARPT, and ΔKLD were generated from hPmel17-l in pCI and have been described (26). The ΔCS point mutant, in which the codons for 468KR469 have been altered to QQ by site-directed mutagenesis, has been described (15); the ΔCS/319–344 deletion construct was a fortuitous construct that was identified while screening for the ΔCS construct and was subsequently subcloned into pCI as for ΔCS.

Metabolic Labeling and Immunoprecipitation—Cells were metabolically labeled with [35S]methionine/cysteine and chased essentially as described (46). Briefly, cells were released from dishes by trypsinization or treatment with PBS, 5 mM EDTA, washed, and then preincubated in suspension in methionine/cysteine-free Dulbecco’s modified Eagle’s medium supplemented with 5% dialyzed fetal bovine serum for 30 min at 37 °C. Cells were then labeled with EasyTag EXPRESS35S Protein Labeling Mix (PerkinElmer Life Sciences) for 30 min, pelleted, and either incubated in chase medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 3 mM methionine, and 3 mM cystine) or directly washed in PBS (for 0 chase). For tunicamycin treatment, cells were pre-treated with 2 μg/ml (2.4 μM) tunicamycin (Calbiochem) for 60 min at 37 °C prior to labeling, and tunicamycin was included in both labeling and chase media. Control cells were treated with equal volumes of dimethyl sulfoxide without tunicamycin. Cell pellets were resuspended in Triton lysis buffer (Tris-buffered saline, pH 7.4, 1% Triton X-100 with protease inhibitors), pre-cleared with protein A- or protein G-Sepharose (GE Healthcare), and immunoprecipitated with antibody prebound to protein A- or protein G-Sepharose. Sepharose pellets were washed, eluted with SDS-PAGE sample buffer, and fractionated by SDS-PAGE along with 14C-labeled markers purchased from GE Healthcare; gels were prepared with 10% acrylamide in most experiments. Dried gels were analyzed by PhosphoImager analysis (GE Healthcare) using ImageQuant software (GE Healthcare). For reimmunoprecipitation, primary immunoprecipitates were eluted by two sequential rounds of boiling in 1% (w/v) SDS, 5 mM 2-mercaptoethanol, 0.5 M Tris, pH 6.8. Eluates were treated with 20 mM iodoacetamide on ice for 20 min and then diluted 10-fold with Triton lysis buffer, and equal aliquots were subjected to secondary immunoprecipitations exactly as detailed above.

Glycosidase Treatments—Immunoprecipitates were treated directly with endoglycosidases after a final wash with phosphate-buffered saline. Denaturation buffer (0.5% (w/v) SDS, 40 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin) was added to protein A-agarose pellets, and the samples were heated to 100 °C for 10 min. After cooling on ice, samples were either mock-treated or treated with endoglycosidases. EndoH (Endo H, New England Biolabs, Ipswitch, MA) and protein N-glycanase F (PNGase F; New England Biolabs) only treatments were according to the manufacturer’s instructions, except that EndoH digestions included 1% Nonidet P-40 in the buffer. Treatment with PNGase F and neuraminidase or with PNGase F and “O-glycosidases” were done using the glycoprotein deglycosylation kit from Calbiochem. Briefly, incubations were done for 4–16 h at 37 °C in 50 mM sodium phosphate buffer, pH 7.0, containing 1% (w/v) Triton X-100 using N-glycanase F and Arthrobacter ureafaciens α2–3,6,8,9-neuraminidase alone for PNGase F neuraminidase or with additional Streptococcus pneumoniae endo-α-N-acetylglactosaminidase, β1,4-galactosidase, and β-N-acetylgalcosaminidase for PNGase F and O-glycosidases. In parallel experiments not shown, pellets were treated sequentially with N-glycanase F, neuraminidase, and the mixture of endo-α-N-acetylglactosaminidase, β1,4-galactosidase, and β-N-acetylgalcosaminidase. At the end of the incubations, samples were diluted into SDS-PAGE sample buffer and analyzed by SDS-PAGE and PhosphorImager analysis. For immunoblotting, cells were lysed directly in denaturation buffer (2.5 × 106/ml) and then treated exactly as described for immunoprecipitates above before fractionation by SDS-PAGE and immunoblotting.
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**Immunoblotting**—Western blotting was done as described (46). Briefly, whole cell lysates prepared from equal numbers of different cells were prepared by heating cells to 100 °C for 10 min in SDS-PAGE sample buffer. Samples were fractionated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), and membranes were blocked and probed with primary antibodies diluted in buffer containing 5% milk. Bound antibody was detected with the use of alkaline-phosphatase-conjugated goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA), ECF detection system (GE Healthcare), and PhosphorImager analysis with ImageQuant software (GE Healthcare).

**Immunofluorescence Microscopy**—Cells grown on coverslips were fixed and permeabilized as above before labeling with primary and fluorochrome-conjugated secondary antibodies as described (47). Most experiments used Alexa488- and Alexa594-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Molecular Probes/Invitrogen). In experiments comparing labeling by two monoclonal antibodies, we used isotype-specific secondary antibodies (HMB45 is IgG1; HMB50 is IgG2a; and NKI-beteb is IgG2b) purchased from Jackson ImmunoResearch and conjugated to Alexa488 or Alexa594 using labeling kits from Molecular Probes/Invitrogen. Cells were analyzed on a Leica Microsystems DM IRBE microscope (Bannockburn, IL), and digital images at different z planes, separated by 0.2-μm steps, were captured using a Hamamatsu ORCA camera (Malvern, PA) and Improvision OpenLab software (Lexington, MA). Images were deconvolved using the OpenLab Volume Deconvolution module. Most images shown are deconvolved images of either a single z plane or a merged image of three to four deconvolved z planes. Final images were processed using Adobe Photoshop software (San Jose, CA).

**Flow Cytometry**—MNT-1 cells grown in 10-cm dishes were suspended using phosphate-buffered saline, 5 mM EDTA, and then washed first into medium and then into FACS buffer (FB: PBS, 5% fetal bovine serum, 0.5 mM EDTA). To detect cell surface molecules, cells were incubated on ice with primary rabbit antibodies (1–2 μg/ml) and mouse anti-tubulin (1:1000) diluted in FACS buffer for 30–60 min and then washed twice at 0 °C before subsequent incubation with Alexa488-conjugated anti-mouse Ig and phycoerythrin (PE)-conjugated anti-rabbit Ig. After washing twice at 4 °C, cells were either analyzed immediately or fixed with 2% formaldehyde prior to subsequent analysis. For internalization experiments, cells were incubated at 37 °C for 30 min in medium containing the same concentration of primary rabbit antibodies or NKI-beteb (1:500), HMB45 (1:50), or XD5.A11 (2 μg/ml). Cells were then fixed with 1% (w/v) formaldehyde in PBS for 15 min at room temperature. Cells were permeabilized by one wash with FB containing 0.5% saponin and then incubated with PE-conjugated anti-rabbit or anti-mouse Ig in the same buffer for 30 min on ice. After washing twice, cells were analyzed immediately or after a second fixation as above. For intracellular labeling, unlabeled cells were fixed and permeabilized as above before labeling with primary (aPmel-1 and 1:500 anti-Tac as a control) and secondary (PE-conjugated anti-rabbit Ig) antibodies. Cells were analyzed on a FACSCalibur using CellQuest Pro software (BD Biosciences).

**Electron Microscopy**—MNT-1 cells were fixed with 0.2% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde, processed for cryosectioning, and immunogold labeled using protein A gold conjugated to 10- and 15-nm gold particles essentially as described previously (14). Cells were analyzed on a Philips TEM/CM120 transmission electron microscope (Eindhoven, The Netherlands), and acquisitions were made with a Keen-View digital camera (Soft Imaging System, Germany).

**RESULTS**

**Monoclonal Antibodies HMB50 and NKI-beteb Bind to the PKD Domain Contained within the Mα Fragment**—Pmel is recognized by three widely used, commercially available monoclonal antibodies as follows: HMB45 (48–50), HMB50 (49, 50), and NKI-beteb (36). The epitope for HMB45 requires modification of linked oligosaccharides by sialic acid (25, 29, 30) and was recently mapped to a region of internal repeats (RPT) encompassed by Mα within the Pmel luminal domain (51) (see Fig. 1a). By contrast, the epitopes for HMB50 and NKI-beteb were mapped to a region encompassed by Mβ, downstream of Mα, by virtue of a reimmunoprecipitation assay (28; see below). To accurately localize the epitopes for these antibodies, we assessed their ability to recognize targeted Pmel deletion constructs expressed in HeLa cells. Immunoreactivity was compared with that of rabbit antibodies generated against specific peptide regions of Pmel (Table 1). Pmel constructs were generated bearing deletions within each of four luminal sub-domains (26) (Fig. 1a) as follows: an N-terminal region (NTR), a downstream region homologous to a polycystic kidney disease-1 repeat (PKD), and RPT are all encompassed by Mα, while the C-terminal half of KLD lies downstream of the proprotein convertase cleavage site (CS) and is thus encompassed by Mβ (only the C-terminal half of KLD was deleted, as constructs with more extensive KLD deletions were unstable and thus undetectable by any of the antibodies). In addition, ΔCS, in which Gly88Lys469 was altered to QQ to eliminate the CS (15), was analyzed to assess any requirement for proteolytic processing in antibody recognition. Each mutant and wild-type Pmel was expressed by transient transfection in HeLa cells, and cells were analyzed in three ways.

Cells were first analyzed by immunoprecipitation following metabolic pulse labeling with [35S]methionine/cysteine (Fig. 1, b and c). As expected, all constructs tested were immunoprecipitated by rabbit antibody aPep13h directed to a peptide encompassing the Pmel C terminus within the cytoplasmic domain. Similarly, all constructs except ΔNTR were recognized by aPmel-N to the N terminus, and all constructs except ΔRPT were recognized by aPmel-I directed to a peptide corresponding to residues 326–344 within the RPT region (Fig. 1, b and c). Importantly, whereas both HMB50 and NKI-beteb effectively immunoprecipitated constructs lacking RPT, CS, or KLD, neither antibody immunoprecipitated ΔPKD above background levels (Fig. 1b). Both antibodies weakly immunoprecipitated ΔNTR above background (Fig. 1c), suggesting that the primary determinant for both HMB50 and NKI-beteb lies in the PKD. Indeed, HMB50 and NKI-beteb also detect the Pmel PKD domain in the context of a chimeric protein lacking other Pmel-derived domains (data not shown). Moreover, both HMB50...
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and NKI-beteb bind to secreted forms of Mα that are released from the membrane-tethered Mβ fragment (see Fig. 2). Deletion of NTR likely reduces binding by these antibodies indirectly, perhaps by influencing PKD integrity. This would be consistent with variable effects of NTR deletion on the PKD-dependent localization of Pmel to internal membranes of multivesicular bodies (26, 51). Together, these experiments show that HMB50 and NKI-beteb recognize a region within the PKD but not within Mβ as previously reported (28).

To confirm and extend these results, transfected cells were analyzed by IFM. Cells expressing ΔKLD (Fig. 1e), ΔCS, or wild-type Pmel (data not shown) were recognized by all three monoclonal antibodies. Cells expressing ΔRPT were recognized by NKI-beteb but not by HMB45 (Fig. 1e), confirming reactivity of HMB45 with the RPT region as reported previously (51). Consistent with the immunoprecipitation results, cells expressing ΔPKD were recognized by HMB45 but not by NKI-beteb (Fig. 1e), confirming that NKI-beteb detects a determinant within PKD. Cells expressing ΔNTR were recognized by all three antibodies using this technique (Fig. 1e), confirming that the NTR is not the primary determinant recognized by NKI-beteb. Note, however, that labeling was not as extensive for NKI-beteb as for HMB45, confirming that NTR deletion compromises the ability of these antibodies to detect the PKD. In all analyses, HMB50 showed identical reactivity to NKI-beteb (data not shown), indicating that it too recognizes an epitope within the PKD of human Pmel.

To confirm the determinant recognized by HMB45, lysates of transfected HeLa cells were analyzed by immunoblotting (Fig. 1d) because HMB45 can detect Pmel by this technique (29) but not by immunoprecipitation. Two additional Pmel variants were included in these analyses; Pmel-s is the product of the shortest of the natural splice variants of Pmel and lacks residues 373–414 within RPT of the longest form (23), and ΔCS/319–344 has a mutated cleavage site (as in ΔCS) and bears a fortuitous deletion of residues 319–344 within RPT (Fig. 1a). Although all constructs analyzed were detected with αPep13h to the Pmel C terminus, HMB45 failed to detect any signal from cells expressing either ΔRPT or ΔCS/319–344 (Fig. 1d). This confirms and extends previous mapping of the HMB45 epitope (51), indicating that the epitope lies between residues 328 and 344. Interestingly, only weak bands were detected in cells expressing ΔPKD and ΔNTR, both of which mature slowly by pulse/chase analyses and are defective in transport to late endosomes in HeLa cells (26). This supports the requirement for post-ER processing in generation of the HMB45 epitope (29, 30). Finally, whereas Pmel-s was detected normally by HMB45, the major detected product migrated slightly slower by SDS-PAGE than that generated from the membrane-tethered Mβ fragment (see Fig. 2). Deletion of NTR likely reduces binding by these antibodies indirectly, perhaps by influencing PKD integrity. This would be consistent with variable effects of NTR deletion on the PKD-dependent localization of Pmel to internal membranes of multivesicular bodies (26, 51). Together, these experiments show that HMB50 and NKI-beteb recognize a region within the PKD but not within Mβ as previously reported (28).

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Together, these studies demonstrate that HMB45, HMB50, and NKI-beteb detect epitopes contained within Mα. Thus their detection of premelanosomes can be explained by their reactivity with Mα fibrils alone.

Pmel Antibodies Coimmunoprecipitate Mα with Mβ from Cell Lysates But Not from Supernatants—The previously reported assignment of HMB50 and NKI-beteb epitopes to a
region within Mβ was based on the reprecipitation of Mβ with these antibodies following elution from primary immunoprecipitates (28). This technique failed to account for covalent disulfide linkage of cleaved Mα and Mβ (5). Indeed, following metabolic pulse/chase analysis of human MNT-1 melanoma cells, identical band patterns are visualized at each time point by immunoprecipitation of cell lysates with HMB50, NKI-beteb, and αPmel-N or αPep13h to the N or C terminus of Pmel, respectively (Fig. 2, a–e). As expected, secreted Pmel, which has been shown to correspond to Mα (5, 24), was immunoprecipitated by αPmel-N, HMB50, and NKI-beteb, but not by the C terminus-directed αPep13h (Fig. 2, a–e). To affirm that the secreted form recognized by all three antibodies corresponds to the same polypeptide, we used a reprecipitation assay. Secreted polypeptides were immunoprecipitated with HMB50, NKI-beteb, or a control mAb from supernatants of cells that had been pulse-labeled and chased for 2 or 4 h, and material was released from immunoprecipitates by boiling in 1% SDS as described under “Experimental Procedures.” Released material was diluted and subjected to reimmunoprecipitation. As shown in Fig. 2f, the secreted Pmel band was reprecipitated by αPmel-N and HMB50, but not by αPep13h, confirming that it bears the Pmel N terminus and PKD domain but not the C terminus. By contrast, full-length Pmel from cell lysates was reprecipitated by all three antibodies (data not shown). Together, these data confirm that HMB50 and NKI-beteb detect epitopes present within Mα and not within Mβ.

One anti-Pmel antibody showed a distinct pattern of recognition from the others in metabolic pulse/chase analysis of MNT-1 cells. αPmel-l, directed to residues 326–344, only detected the full-length, core-glycosylated “P1” form of Pmel in cell lysates and did not detect either the Golgi-modified, EndoH-resistant “P2” form, the post-Golgi cleavage products Mα and Mβ, or secreted Mα (Fig. 2e). Similarly, αPmel-I failed to reprecipitate Mα eluted from HMB50 or NKI-beteb (Fig. 2f). This indicates that αPmel-I only detects the precursor form of Pmel. Interestingly, the peptide to which αPmel-I was generated overlaps nearly completely with that required for HMB45 reactivity (Fig. 1d). Given that HMB45 reactivity also requires O-glycosylation and subsequent modification with sialic acid, we reasoned that αPmel-I reactivity might be ablated by O-glycosylation of the peptide, and thus should faithfully detect only unmodified Pmel. Experiments described below suggest that this is indeed the case.

Antibodies to Immature Forms of Pmel Do Not Detect Stage II Melanosomes—We and others have shown that HMB45, HMB50, and NKI-beteb detect striated stage II melanosomes by electron microscopy of pigmented melanocytes and melanoma cells (14, 15, 36), but that antibodies to the Pmel C terminus recognize neither stage II melanosomes (14) nor Pmel-containing fibrils in melanocytes or transfected HeLa cells (15). By
contrast, Hearing and co-workers (16, 25, 28, 38) report that labeling by the Pmel mAbs in IFM analyses of MNT-1 cells overlaps that by antibodies to the C terminus and to a region encompassed by RPT that, like αPmel-I, is rendered unreactive by O-glycosylation. To directly test antibody overlap, we performed a comparable series of two-color IFM analyses of MNT-1 cells (Fig. 3). To balance preservation of subcellular structures with immunodetection, cells were fixed with either 2 or 6% formaldehyde, and to improve resolution, images from sequential z-stacks were subjected to deconvolution analyses as described under “Experimental Procedures.” Consistent with all previous conclusions, all three mAbs detected overlapping punctate structures within MNT-1 cells (Fig. 3A, panels a–i) that likely correspond to stage II melanosomes (14). The reactivity of these structures with HMB50 and NKI-beteb decreased with increasing fixative concentration such that HMB45 detected puncta not observed with these two antibodies, but nearly all structures labeled by these antibodies also labeled with HMB45 under all conditions. By contrast, neither αPep13h to the C terminus, αPmel-N to the N terminus, nor αPmel-I detected these same structures (Fig. 3B, panels a–i). Rather, the anti-peptide antibodies detected tubular structures throughout the cell body that were stabilized with higher concentrations of fixative and particularly concentrated in the perinuclear region. They appeared more punctate, but equally widely distributed, upon fixation with lower fixative concentrations (not shown). In addition, αPep13h detected a few punctate structures that did not overlap with the majority of puncta detected by NKI-beteb (Fig. 3B, panels a–c) but partially overlapped with the early endosomal marker EEA1 (not shown); these structures likely represent stage I melanosomes (5, 14, 15). αPmel-I, which detects only the core glycosylated P1 form of Pmel (Fig. 2), did not
detect these puncta (Fig. 3B, panels d–f), consistent with its reactivity only with pre-Golgi Pmel isoforms. Together, these data indicate that antibodies that detect biochemically immature forms of Pmel at steady state do not significantly label stage II melanosomes. The failure of αPmel-N to detect stage II melanosomes at steady state, despite the detection of αPmel-N reactivity on purified fibrils (15), might reflect the rapid processing of Mr to smaller fragments within stage II melanosomes (see Ref. 16 and below).

To define the compartments in which Pmel is detected by these antibodies, we compared their overlap with markers of different compartments. We have shown previously by immunoelectron microscopy that αPep13h detects Pmel on Golgi membranes, the trans-Golgi network, and the limiting membrane of stage I melanosomes in MNT-1 cells (14), consistent with the perinuclear labeling observed by IFM (Fig. 3B, panels a–c) that overlapped significantly with Golgi markers. Interestingly, whereas αPmel-I does not label the perinuclear structures as extensively, the tubular network observed throughout the cell body overlapped significantly with both calnexin, a component of the ER quality control system (Fig. 4, a–c), and ERGIC-53, which concentrates primarily in ER-to-Golgi intermediates (Fig. 4, d–f). Consistent with the IFM analyses, electron microscopy analyses of immunogold-labeled cryosections of MNT-1 cells showed αPmel-I labeling over tubulovesicular membranes associated with one face of stacked Golgi cisternae (Fig. 5a), in which αPmel-I labeling showed significant overlap with ERGIC-53 or the KDEL receptor (KDEL-R, which also cycles between the ER and Golgi; Fig. 5, d–f). Additional labeling was observed on tubular membranes with morphological hallmarks of ER (Fig. 5, b and c). Some of these membranes lacked ribosomes, perhaps explaining the incomplete overlap of αPmel-I or αPep13h labeling with rough ER markers such as BiP and calnexin (here and see Ref. 16). Together, these data indicate that αPep13h and αPmel-I detect a significant cohort of Pmel that accumulates in the ER and pre-Golgi intermediates but that are not associated with fibrillar stage II melanosomes.

Only Golgi-modified Forms of Pmel Are Detected at the Cell Surface and Internalized—Full-length P1 Pmel or Pmel lacking O-linked oligosaccharides have been reported to copurify and colocalize with clathrin adaptors AP-1 and AP-2 in the cell periphery, supporting the existence of Pmel lacking Golgi modifications in pre-melanosomal compartments (25, 38). AP-2 is concentrated in clathrin-coated pits at the plasma membrane,
where it facilitates endocytosis (39). Pmel undergoes rapid endocytosis in mouse melanocytes and transfected HeLa cells (12, 52). Thus, if immature forms of Pmel were indeed present in AP-2-containing structures, they should be detectable at the plasma membrane and subject to endocytosis. To test these predictions, we used quantitative flow cytometric assays. Surface Pmel was detected on MNT-1 cells in suspension by antibody labeling on ice. As shown in Fig. 6a and quantified in Fig. 6b, αPmel-N detects a significant cohort of Pmel17 at the surface of MNT-1 cells, the signal obtained with αPmel-I was as low as that obtained with the negative control αPep13h. High levels of NKI-beteb and low levels of HMB45, which has very low affinity for native Pmel, based on its inability to immunoprecipitate Pmel, were also internalized (Fig. 6d). Although αPmel-I failed to either bind to the cell surface or internalize, it bound avidly to intracellular Pmel after fixation and permeabilization of MNT-1 cells (Fig. 6e). Together, these data indicate that although a cohort of Pmel cycles through the plasma membrane of MNT-1 cells, immature, unprocessed forms of Pmel do not significantly contribute to this cohort.

Detectable Pmel17 Fragments in Melanosomes Are O-Glycosylated and Modified by Sialic Acid—N- and/or O-linked glycans on mature forms of Pmel bear sialic acid residues that are required for HMB45 reactivity (29, 30). To better define the cohort of Pmel with such modifications, we tested the effect of a set of glycosidases on electrophoretic migration (by SDS-PAGE) and antibody reactivity of the different Pmel isoforms. Mobility changes induced by EndoH cleavage defined high mannose N-linked oligosaccharides that have not been modified by medial Golgi N-acetylgalacosaminyltransferase. PNGase F cleavage defined N-linked oligosaccharides regardless of their state of modification. α2–3,6,8,9-Neuraminidase cleavage defined terminal sialic acid residues on either N- or O-linked oligosaccharide chains. Finally, cleavage by a combination of neuraminidase with β1,4-galactosidase, endo-α-N-acetylgalactosaminidase, and β-N-acetylgalactosaminidase (collectively referred to here as O-glycanases) defined a common form of O-linked oligosaccharide.

MNT-1 cells were metabolically labeled with [35S]methionine and chased for 1–2 h to accumulate labeled P1, P2, Mα, and Mβ within cell lysates. Pmel isoforms were immunoprecipitated from cell lysates with αPep13h and then either mock-


treated or treated with EndoH (H), PNGase F alone (F), PNGase F with neuraminidase (FN), or PNGase F with neuraminidase and O-glycosidases (FON). As shown in Fig. 7 (left panel), each treatment resulted in a characteristic shift for each band. As shown previously (5), the P1 band is completely sensitive to digestion by EndoH or PNGase F, with molecular mass reduced by ≈10 kDa (to P1’H) consistent with removal of three to four core N-linked glycans. No additional reductions in M were observed upon additional glycosidase treatment. Together, these data indicate that P1 represents core N-glycosylated Pmel in the ER, cis-Golgi, and/or intermediate compartment, and is not significantly modified by O-glycosylation.

All other bands were predominantly EndoH-resistant. The M, ~ 28,000 Mβ band was reduced in M, by ≈3,500 to Mβ’ F by PNGase F but not EndoH, consistent with a single N-linked glycan that is fully modified to the complex form in the Golgi. The lack of additional effects by neuraminidase or O-glycosidases indicates that Mβ is not modified by at least conventional O-linked oligosaccharides. Both P2 and Mα were modestly reduced in M, by EndoH but more dramatically by PNGase F (to P2’F and Mα’F), consistent with 4 and 3 N-linked glycans, respectively, all but one of which are modified to the complex type. Additional treatment with neuraminidase increased the M, relative to PNGase F treatment alone, for both P2 and Mα (to P2’FN, Mα’FN), reflecting loss of sialic acid residues from O-linked oligosaccharides and consequent decreased electrophoretic migration toward the cathode. Consistently, additional treatment with O-glycosidases dramatically reduced the M, of both P2 and Mα (to P2’FON and Mα’FON). The changes in migration did not reflect a loss of enzyme activity within the mixtures, because identical results were observed when samples were treated consecutively with each enzyme rather than concomitantly (data not shown). No Pmel forms bearing only O-linked oligosaccharides without complex N-linked oligosaccharides were detected. These data indicate that post-ER forms of Pmel possess both complex-type N-linked glycans and sialylated O-linked glycans. Interestingly, the PNGase F- and O-glycosidase-treated P2’FON continues to migrate slower than deglycosylated P1’H (M, ~ 125,000 for P2’FON, M, ~ 97,000 for P1’H in the experiment shown), suggesting that it retains additional post-ER modifications that are not removed by the O-glycosidases used here.

What modifications are present on Pmel isoforms in melanosomes? HMB45 reacts predominantly with a series of bands with M, ~ 40,000 that are exclusively found in the detergent-insoluble fraction of pigment cells (12, 29) and that cofractionate with mature melanosomes by subcellular fractionation (16, 28). As shown in Fig. 7 (upper right panel), these bands are not

FIGURE 6. Pmel isoforms detected by αPmel-I do not access the cell surface. a and b, MNT-1 cells in suspension were labeled on ice with the indicated antibodies and PE-conjugated anti-rabbit Ig and analyzed by flow cytometry as described under “Experimental Procedures.” a, fluorescence intensity signal relative to cell number is shown for a representative experiment; b, median fluorescence intensity for samples from two experiments performed in duplicate are indicated graphically along with standard deviation. c and d, MNT-1 cells in suspension were incubated for 30 min at 37 °C with the indicated antibodies, then fixed, permeabilized, and labeled with PE-conjugated anti-rabbit Ig and Alexa488-conjugated anti-mouse Ig prior to analysis by flow cytometry. c, results from a representative experiment; d, median fluorescence intensity for samples from two experiments performed in duplicate is indicated graphically along with standard deviation. The control mAb (Ctl. mAb) was XD5.A11, which recognizes human leukocyte antigen class II β chains. e, MNT-1 cells in suspension were fixed and permeabilized prior to labeling with αPmel-I or a control rabbit antibody (Ctl. Ab; anti-Tac). Representative of three experiments performed in duplicate.
affected by treatment of MNT-1 whole cell lysates with EndoH or PNGase F but are completely lost upon subsequent treatment with neuraminidase with or without O-glycanase. This repeats previously published data (25, 29) and indicates that HMB45 recognizes an epitope requiring sialylated glycans. Immunoblotting of the same samples with HMB45 reveals no detectable low Mr bands in cell lysates that are untreated or treated with EndoH, PNGase F, or PNGase F and neuraminidase (Fig. 7 upper right panel); a weak band corresponding to P1 was observed in other experiments under conditions that favored detection of high molecular weight material, but no other bands were ever observed. However, additional treatment with O-glycanase unveils a novel reactive band with Mr 26,000, consistent with that expected for a deglycosylated PKD-RPT fragment (predicted mass of 29 kDa). Because no comparably strong band was observed at all in untreated cell lysates, this band must correspond to the deglycosylated form of the HMB45-reactive bands observed in untreated cell lysates. The detection of this band only after deglycosylation of cell lysates supports the notion that only fully O-glycosylated forms of Pmel are present in melanosomes.

**Glycosylation and Antibody Reactivity of Pmel in Cells That Lack O-Glycosylation**—To confirm the relationship between Pmel O-glycosylation and epitope recognition by αPmel-I and HMB45, we exploited a variant CHO cell line, idld14. idld14 cells lack UDP-galactose- and UDP-N-acetylgalactose-4-epimerase activity (45), which under normal growth conditions are required to initiate O-glycosylation by generating sufficient cellular levels of UDP-N-acetylglucosamine for transfer to target serine and threonine residues (53). Thus, these cells lack all O-glycosylated proteins under normal growth conditions (45). Wild-type CHO-K1 (WT CHO) or idld14 cells were transfected with a Pmel expression vector and analyzed by IFM with HMB45 (a and c) or αPmel-I (b and d). Each pair of panels is from the same field, and images of labeling with each antibody are taken at identical exposures in both cell lines. Note the absence of HMB45 reactivity and the appearance of extensive labeling of the cell surface and intracellular puncta with αPmel-I in idld14 cells. By contrast, αPmel-I labels a reticular network (indicative of the ER) in cells expressing high levels of Pmel but very little, other than background nuclear labeling, in cells expressing low levels of Pmel (Fig. 8, c–f). wild-type CHO-K1 cells (CHO WT; a and b) or idld14 cells (c and d) were transfected with full-length Pmel expression vector and analyzed by IFM with HMB45 (a and c) or αPmel-I (b and d). Each pair of panels is from the same field, and images of labeling with each antibody are taken at identical exposures in both cell lines. Note the absence of HMB45 reactivity and the appearance of extensive labeling of the cell surface and intracellular puncta with αPmel-I in idld14 cells. By contrast, αPmel-I labels a reticular network (indicative of the ER) in cells expressing high levels of Pmel but very little, other than background nuclear labeling, in cells expressing low levels of Pmel (Fig. 8, c–f). wild-type CHO-K1 cells (CHO WT; a and b) or idld14 cells (c and d) were transfected with full-length Pmel expression vector and analyzed by IFM with HMB45 (a and c) or αPmel-I (b and d). Each pair of panels is from the same field, and images of labeling with each antibody are taken at identical exposures in both cell lines. Note the absence of HMB45 reactivity and the appearance of extensive labeling of the cell surface and intracellular puncta with αPmel-I in idld14 cells. By contrast, αPmel-I labels a reticular network (indicative of the ER) in cells expressing high levels of Pmel but very little, other than background nuclear labeling, in cells expressing low levels of Pmel (Fig. 8, c–f). wild-type CHO-K1 cells (CHO WT; a and b) or idld14 cells (c and d) were transfected with full-length Pmel expression vector and analyzed by IFM with HMB45 (a and c) or αPmel-I (b and d). Each pair of panels is from the same field, and images of labeling with each antibody are taken at identical exposures in both cell lines. Note the absence of HMB45 reactivity and the appearance of extensive labeling of the cell surface and intracellular puncta with αPmel-I in idld14 cells. By contrast, αPmel-I labels a reticular network (indicative of the ER) in cells expressing high levels of Pmel but very little, other than background nuclear labeling, in cells expressing low levels of Pmel (Fig. 8, c–f).
observed in \textit{ldlD14} cells with the same antibody (Fig. 8, \textit{b} and \textit{d}). Thus, when Pmel is not modified by O-glycosylation, the \textalpha Pmel-I epitope is expressed later as well as early in the secretory pathway (although note that the puncta were less intense than the plasma membrane labeling, see below). Consistently, immunoblotting of lysates of Pmel-transfected cells with HMB45 reveals the expected \( M_r \sim 40,000 \) band and a faint \( M_r \sim 95,000 \) band in CHO WT cells but no reactivity at all in \textit{ldlD14} cells (Fig. 8 \textit{e}, \textit{middle}), despite equivalent detection of full-length P1 and cleaved M\( \beta \) fragments in both cell lysates by \textalpha Pep13h (Fig. 8 \textit{e}, \textit{top}). By contrast, immunoblotting of \textit{ldlD14} cell lysates with \textalpha Pmel-I revealed a smear of reactivity from \( M_r \sim 20,000 \) to \( M_r \sim 80,000 \) that was absent from lysates of either CHO WT or MNT-1 cells (Fig. 8 \textit{e}, \textit{bottom}). These data confirm that HMB45 reactivity requires O-glycosylation and that \textalpha Pmel-I reactivity is ablated by O-glycosylation. Interestingly, the HMB45-reactive bands in MNT-1 cell lysates were much more intense than those in CHO WT lysates, despite lower Pmel precursor expression detected by \textalpha Pep13h. This suggests that fully processed Pmel fragments are less protected in CHO WT than in MNT-1. The lack of a similar band and the presence of a smear detected by \textalpha Pmel-I in \textit{ldlD14} cells, consistent with the weak labeling by this antibody of intracellular puncta relative to the cell surface, suggests that even this minimal protection of the fragment requires O-glycosylation.

To determine whether Pmel bears modifications other than N- or O-linked glycosylation, transfected CHO WT and \textit{ldlD14} cells were analyzed by metabolic pulse/chase and immunoprecipitation analyses. As shown in Fig. 8\textit{f}, Pmel matures to a high \( M_r \) P2 form and to cleaved M\( \alpha \) and M\( \beta \) forms by 2 h in both cell lines. However, the P2 and M\( \alpha \) forms migrate faster in \textit{ldlD14} than in CHO WT, consistent with a loss of mass contributed by O-linked glycans. Moreover, both
bands show increased migration upon treatment with PNGase F in both cell types, but whereas the treated P2 and P1 bands in CHO WT cells remain distinct, the P2 and P1 bands from ldlD14 collapse into a single band. This indicates that removal of both O- and N-linked oligosaccharides eliminates the migration differences between the core glycosylated P1 form and the mature P2 form in CHO cells.

**Pmel Trafficking Is Unaffected by Glycosylation**—Although the HMB45 and αPmel-1 reactivity of Pmel in ldlD14 cells was expected based on the data described in Figs. 2, 5, and 6, the processing of Pmel to Mα and Mβ fragments in these cells detected by immunoblotting with αPep13h (Fig. 8e, top) and by metabolic pulse/chase analysis (Fig. 8f) was surprising. Pmel cleavage to Mα and Mβ requires proprotein convertase activity in an acidic post-Golgi compartment (5, 15) and correlates with transport to multivesicular endosomes in HeLa cells (26). To determine whether Pmel trafficking requires O-glycosylation, Pmel localization was assessed in transfected ldlD14 cells by IFM using the O-glycosylation-insensitive NK1-beteb antibody. In CHO WT cells, Pmel was detected in vesicular structures throughout the cell body that were closely apposed to structures labeled for the lysosomal limiting membrane protein LAMP-1 (Fig. 9, a–c). This pattern is reminiscent of that observed for Pmel expressed in HeLa cells, in which Pmel localizes to the intraluminal membranes of multivesicular late endosomes (5, 26), suggesting that Pmel localizes similarly in CHO cells. LAMP-1-containing structures in ldlD14 cells were larger and more irregularly shaped than those in CHO WT cells (Fig. 9e), consistent with known functional requirements for O-glycosylation of lysosomal membrane proteins (54). In cells expressing moderate levels of Pmel, a cohort of NK1-beteb-reactive Pmel localized closely apposed to these LAMP-1-containing structures, although a significant cohort was found in separate punctate structures and the plasma membrane (Fig. 9, d–f). In cells overexpressing Pmel, a larger cohort of NK1-beteb-reactive Pmel colocalized with LAMP-1-containing structures, which were often more clustered in the perinuclear region and reduced in LAMP-1 content (Fig. 9, g–i). Together, these data suggest that Pmel reaches late endosomes/lysosomes in ldlD14 cells and might influence their physiology. This would imply that whereas O-glycosylation is required for stabilizing processed Pmel fragments derived from the RPT region, it is not required for transport of remaining Pmel fragments to late endosomal organelles.

If O-linked oligosaccharides are not necessary for transport, is there a requirement for N-linked oligosaccharides? To test this, MNT-1 cells were treated with tunicamycin, which inhibits transfer of core N-linked glycans from the dolichol-linked intermediate to asparagine acceptor sites on polypeptides. Because tunicamycin affects cell viability, transport was assessed indirectly by assaying for O-glycosylation and proteolytic maturation to Mα and Mβ. As shown in Fig. 9j, tunicamycin resulted in the expected decrease in M, for P1, P2, M0, and Mβ bands. Nevertheless, P2, M0, and Mβ were each generated to a similar extent in tunicamycin-treated cells and untreated controls, indicating that Pmel was O-glycosylated and transported to a compartment in which it was cleaved by a proprotein convertase. These data indicate that N-linked oligosaccharides are not required for Pmel progression through the Golgi to multivesicular endosomes.

**DISCUSSION**

Pmel17 is a critical component of the amyloid-like fibrils of melanosomes, but how it is delivered to melanosome precursors has been controversial. Here we combine epitope detection using different anti-Pmel antibodies with analyses of Pmel glycosylation, localization, and processing to show that only...
luminal products of post-Golgi-cleaved and Golgi-modified Pmel are detected in stage II melanosomes. Our data raise questions about the interpretation of previous results postulating the existence within these organelles of separate pools of Pmel, some of which lack Golgi modifications, and have important implications for the mechanism of Pmel fibril formation.

No Evidence for Immature Glycosylated Forms of Pmel or Precursors to Cleaved Products in Melanosomes—We have previously shown that post-Golgi cleavage of Pmel by a proprotein convertase is required to generate fibril-forming Ma fragments (5, 15, 26), but it has been argued that uncleaved forms of Pmel with unprocessed N-linked oligosaccharides are found in stage II melanosomes or colocalized with endocytic and endosomal adaptors AP-1 and AP-2 in post-Golgi compartments (16, 25, 28, 38). Here we provide evidence that this is not the case. First, we unequivocally map epitopes for monoclonal antibodies HMB-50 and NKI-beteb to the PKD domain within Ma (Fig. 10a), and not to a region downstream of Ma as suggested previously (28). That these antibodies and HMB45, which recognizes a sialylated form of the RPT domain within Ma (here and see Refs. 25 and 51), all detect stage II melanosomes by electron microscopy (14, 36) is consistent with the identification of Ma-derived fragments in fibril preparations of stage II melanosomes (15, 16). Second, we show using IFM and deconvolution analyses that labeling of melanocytic cells by all three of these monoclonal antibodies does not overlap labeling by rabbit antibodies to the N or C terminus of Pmel or to the αPmel-I epitope masked by O-glycosylation. These data indicate that only O-glycosylated forms of Pmel lacking the N and C termini exist within stage II melanosomes, consistent with Golgi-modified Ma fragments spanning the PKD and RPT regions. We speculate that earlier observations of colocalization of immature Pmel detected by similar antibodies with stage II melanosomes or endosomes by IFM (16, 25, 28, 38) reflected nonspecific over-precipitation, they do not significantly label early secretory compartments by IFM analyses. We speculate that this is a consequence of the high epitope density of mature, polymerized, and antibody-reactive Pmel isoforms on fibrils relative to the predicted lower density within the ER and Golgi. Indeed, both antibodies are capable of detecting Pmel that is accumulated within the ER when it is depleted from later compartments (12). Confirmation of this speculation will require a more effective means of quantitatively extracting Pmel from fibrils to accurately assess its concentration within them.

Role of Glycosylation in Pmel Function—That Pmel harbors both N- and O-linked glycans is well accepted. Our data and those previously published (25) show that sialylated O-linked glycans are added to at least two distinct sites within the RPT domain, within regions spanning residues 328–344 and 393–414 (Fig. 10a). Although there is no consensus site for serine or threonine modification by the initial αGalNAc linkage, acceptor sites tend to be rich in serine, threonine, and proline residues (58). These residues are present throughout the RPT domain, suggesting that O-glycan modifications might occur throughout the domain (Fig. 10a). The RPT domain has been shown to be required for the formation of amyloid-like Pmel fibrils by immunoprecipitation, they do not significantly label early secretory compartments by IFM analyses. We speculate that this is a consequence of the high epitope density of mature, polymerized, and antibody-reactive Pmel isoforms on fibrils relative to the predicted lower density within the ER and Golgi. Indeed, both antibodies are capable of detecting Pmel that is accumulated within the ER when it is depleted from later compartments (12). Confirmation of this speculation will require a more effective means of quantitatively extracting Pmel from fibrils to accurately assess its concentration within them.

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fibrils in late endosomal compartments of HeLa cells (26, 51), but the extensive modification of this domain by O-glycans would likely, although not necessarily, impede the formation of the highly compact cross β-sheet structure that underlies the amyloid core (59). Perhaps the RPT domain functions as a regulator of fibril formation rather than a structural component.

Analyses of Pmel migration following deglycosylation with a combination of enzymes demonstrated two features of the heterogeneity of Pmel glycosylation. First, we detected no evidence for the existence of Pmel glycoforms bearing O-linked oligosaccharides with immature N-linked oligosaccharides. That such glycoforms exist was suggested by Valencia et al. (25) on the basis of the appearance of a rapidly migrating band reactive with the αPEP25h antibody, which has properties similar to that of αPmel-I and detects only the immature P1 form of Pmel in untreated cell lysates, after treatment with O-glycanase and PNGase F. We interpret their data as representing the novel generation of an αPEP25h-reactive band derived by deglycosylation of the HMB45-reactive bands, analogous to that reactive with αPmel-I in our experiment shown in Fig. 7 (right panels). This interpretation is consistent with the fact that the P1 band was not decreased in intensity after O-glycanase treatment in their experiments (25).

A second feature of glycosylation heterogeneity revealed by our data is that in MNT-1 cells, treatment of the Golgi-modified P2 band with both PNGase F and O-glycanases failed to reduce M1 to that of PNGase F-treated immature P1. These data indicate that mature Pmel bears some modification that is resistant to the combination of O-glycanases used in these experiments. By contrast, in O-glycosylation-deficient ldlD14 cells, PNGase F treatment results in an identical M1 for P2 and P1. This indicates that at least in CHO cells, all of the modifications of mature Pmel can be accounted for by both O- and N-linked glycosylation. To reconcile these data, we speculate that the additional modifications observed in MNT-1 cells are a distinct class of O-glycans bearing linkages other than β1,4-linked galactose and/or β1,6-linked N-acetylgalosamine, such as α-linked N-acetylgalactosamine common in many mucins (58). Whether these modifications are characteristic of melanocytic cells in general or acquired during the melanoma transformation of MNT-1 cells remains to be determined.

What function does glycosylation serve to Pmel? We show here that surprisingly, neither O-glycosylation nor N-glycosylation is required for biosynthetic folding and export from the ER or for downstream trafficking to endocytic compartments in which Pmel is cleaved by a proprotein convertase. On the other hand, the lack of accumulation of αPmel-I-reactive low M1 fragments and the weak detection of αPmel-I reactivity in late endosomes of ldlD14 cells suggests that O-glycosylation may be required for stabilization of Pmel RPT-derived fragments (but not NKI-beteb-reactive PKD-derived fragments) within hydrolytic compartments such as late endosomes, lysosomes, and melanosomes. Such a requirement for stabilization might explain the failure to observe fibrils in lcl8 cells (25), in which only the primary αGalNAc linkage to serine or threonine and early Golgi modifications to N-linked oligosaccharides are expected to occur. We could not verify by electron microscopy analyses whether fibrils formed normally in ldlD14 cells because of low transfection efficiencies, but the lack of stable αPmel-I-reactive low M1 fragments in these cells is consistent with a failure to form fibrils. Together, the data suggest that Pmel glycosylation is required for processes that occur downstream of sorting, including stabilization of fibrils or interactions with other proteins. It will be of interest to determine whether N- or O-linked glycans play a direct or indirect role in fibril formation.

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