The Repeat Domain of the Melanosomal Matrix Protein PMEL17/GP100 Is Required for the Formation of Organellar Fibers*

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Over 125 pigmentation-related genes have been identified to date. Of those, PMEL17/GP100 has been widely studied as a melanoma-specific antigen as well as a protein required for the formation of fibrils in melanosomes. PMEL17 is synthesized, glycosylated, processed, and delivered to melanosomes, allowing them to mature from amorphous round vesicles to elongated fibrillar structures. In contrast to other melanosomal proteins such as TYR and TYRP1, the processing and sorting of PMEL17 is highly complex. Monoclonal antibody HMB45 is commonly used for melanoma detection, but has the added advantage that it specifically reacts with sialylated PMEL17 in the fibrillar matrix in melanosomes. In this study, we generated mutant forms of PMEL17 to clarify the subdomain of PMEL17 required for formation of the fibrillar matrix, a process critical to pigment formation. The internal proline-serine/threonyl-rich repeat domain (called the RPT domain) of PMEL17 undergoes variable proteolytic cleavage. Deletion of the RPT domain abolished its recognition by HMB45 and its capacity to form fibrils. Truncation of the C-terminal domain did not significantly affect the processing or trafficking of PMEL17, but, in contrast, deletion of the N-terminal domain abrogated both. We conclude that the RPT domain is essential for its function in generating the fibrillar matrix of melanosomes and that the luminal domain is necessary for its correct processing and trafficking to those organelles.

Melanoma is one of the most notorious tumors because of its poor prognosis (1). Several groups have raised monoclonal antibodies that specifically recognize melanoma cells, with HMB45 being the most popular and frequently used in clinics for the specific detection of melanocytic tumors (2, 3). HMB45 is now known to identify the product of the human PMEL17 locus, also known as GP100 or SILV. Interestingly, two other antibodies developed for melanoma detection, HMB50 and NKI/beteb, also react with PMEL17 (4–7), although the reactivity patterns between HMB45 and HMB50 or NKI/beteb can be quite different (8, 9). Melanosomes are one type of lysosome-related organelle that have the unique capacity to produce melanin pigment (10) and that progress through four sequential morphological stages as they mature (11). Stage I melanosomes are round, membrane-bound, and electron-lucent vesicles. Stage II melanosomes result from the elongation of those vesicles and the appearance within of distinct fibrillar structures. The production of internal matrix fibers, which discriminate Stage II from Stage I melanosomes, depends on the maturation and trafficking of PMEL17 (12–15). In pigmented cells, melanins are synthesized and deposited on those fibers, resulting in a progressively pigmented internal matrix, at which time the organelles are termed Stage III melanosomes. In highly pigmented tissues, melanin synthesis and deposition continue until there is little or no internal structure visible, at which time they are termed Stage IV melanosomes. Several independent groups have used immunoelectron microscopy to show that HMB45 specifically reacts with the fibrillar matrix in Stage II melanosomes (12–14). We reported recently that PMEL17 produces melanosomal matrix fibers in melanocytic cells in the presence of MART-1 (15). Moreover, even in non-melanocytic cells, when adequately overexpressed, PMEL17 can also produce fibrillar structures within lysosome-like structures (12, 16).

Although PMEL17 is studied widely as a melanoma antigen, as noted above, it also plays a crucial role in normal melanocytes as a structural protein in melanosomes (17, 18). PMEL17 is a type I membrane protein that consists of several domains predicted by homology modeling (see Fig. 1A). SIG is the signal peptide thought to determine the entry of PMEL17 into the secretory pathway prior to its processing and cleavage (19), as detailed below. PKD is the polycystic kidney disease-like domain, which has an immunoglobulin-like folding structure (20). RPT is a domain that contains 10 imperfect repeats of 13 proline-serine/threonyl-rich amino acids (21–23). Each of those 13 amino acid series has been annotated from a to j in Fig. 1A, and there are 26 potential O-glycosylation sites within the RPT domain. PMEL17 shows high homology to proteins such as lysosome-associ-
ated membrane protein (LAMP) and NMB (24), but the RPT domain is unique and specific for PMEL17 (25). KRG is the kringle-like domain, which is a triple disulfide-liked autonomous structural domain found in blood clotting and fibrinolytic proteins (26). It is thought that KRG domains play some role in binding interactions with other proteins necessary for their regulation (20); however, nothing has been reported about the function of the KRG domain in PMEL17. TM is the predicted transmembrane domain. The remaining domains are referred to as the N-terminal domain (NTD), the GAP1, GAP2, GAP3 domains, and the C-terminal domain (CTD). PMEL17 has five potential N-glycosylation sites; however, Asn\textsuperscript{322} in the RPT domain is thought not to be utilized (19, 25). Three of those N-glycosylation sites are located in the NTD, and another is in the GAP3 domain. Four isoforms of PMEL17 are generated by alternative splicing (6, 21), as depicted in Fig. 1A. PMEL17-i is the full-length protein sequence, whereas PMEL17-is and PMEL17-ls have truncated RPT domains. The “i” in PMEL17-i and PMEL17-ls indicates the insertion of 7 additional amino acids in the GAP3 domain immediately following the N-glycosylation site. PMEL17-i is the most abundant form (21).\textsuperscript{4}

Fig. 1B depicts the complex pattern of maturation and processing of PMEL17 (based in part on Refs. 12, 15, 16, 25, 27, and 28). Briefly, P1/P100 (hereafter termed P1) is the major endoplasmic reticulum (ER; or early Golgi)-modified form of PMEL17, which contains high mannos-type (but not complex-type) N-glycans and which is endoglycosidase H-sensitive. A relatively small amount of P1 then undergoes further glycosylation in the Golgi to generate P2/P120 (hereafter termed P2), the late Golgi-modified form, which still has at least one high mannos- and hybrid-type N-glycan and which is largely endoglycosidase H-resistant (12, 27). P2 is then cleaved proteolytically (between Arg\textsuperscript{469} and Gln\textsuperscript{470}) within post-Golgi and/or premelanosomal compartments into M\textalpha{} and M\beta{}/P26 (hereafter termed M\beta{}) fragments. Those two fragments remain linked by a disulfide bond. M\alpha{} is thought to be further processed to generate the striated fibrils seen in Stage II melanosomes, although the putative cleavage site(s) in the M\alpha{} fragment are unknown. HMB45 is thought to react with the processed form of M\alpha{}; however, there is no convincing evidence for this at the molecular level. Although there is no controversy that PMEL17 is found in Stage II melanosomes (12, 14), there is some conflict as to which form of PMEL17 (P1 and/or P2) exists in Stage II melanosomes and how it gets there (12, 14, 27). Several studies have shown that HMB45 reactivity is lost when PMEL17 is digested to remove sialic acids (27, 29, 30), but there is as yet no report utilizing that characteristic to define the precise epitope or its implications to the processing and trafficking of PMEL17.

In this study, we focused on characterizing the domain of PMEL17 involved with fibril formation using HMB45 as a fibril-specific probe. We generated mutants of PMEL17 to determine the domains critical to the formation of fibrils and to assess the correct processing and trafficking of PMEL17 that are essential to its function in melanogenesis. We show that HMB45 reacts preferentially with a detergent-insoluble form of PMEL17 and that its epitope, which contains sialylated O-glycans, is located in the second and third amino acid repeats of the RPT domain. The heterogeneous bands detected by HMB45 staining are due to the multiple cleavage points in the RPT domain. Surprisingly, correct processing and trafficking of PMEL17 to LAMP-2-positive organelles are maintained when the RPT domain or the CTD is deleted, but PMEL17 is missorted when other luminal domain(s) are missing, suggesting that important sorting signals are within that region of PMEL17.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Highly pigmented MNT-1 melanoma cells, unpigmented SK-MEL-28 melanoma cells, and HeLa cells were obtained and cultured as described previously (15).

**Plasmids and Transfection**—The pCI mammalian expression vector was purchased from Promega Corp. (Madison, WI). pCI-Pmel17-i, pCI-Pmel17-l, and pCI-Pmel17-s (containing PMEL17-i, PMEL17-l, and PMEL17-ls, respectively, in the pCI vector) were kind gifts from Dr. Michael S. Marks (University of Pennsylvania, Philadelphia, PA). pCI-Pmel17-is was constructed by subcloning the Nsil-XbaI fragments of pCPl-Pmel17-i into pCI-Pmel17-s. The ΔCS mutation (K468Q and R469Q) in pCI-Pmel17-i, pCI-Pmel17-l, pCI-Pmel17-ls, and pCI-Pmel17-ls was introduced as described previously (16). Similarly, ΔSIG, ΔNTD, ΔPKD, ΔGAP1, ΔRPT, ΔGAP2, ΔKRG, ΔGAP3, ΔTM, ΔCTD, RPTa–i, RPTa–g, RPTa–f, RPTa–e, RPTa–d, RPTa–c, RPTa–b, RPTb–j, RPTc–j, and RPTe–j were constructed using pCI-Pmel17-i as the template. The di-alanine mutation from di-leucine sorting signal at the C terminus of PMEL17-l (L662AA) was also introduced. RPTsa–i and RPTsa–h were constructed using pCI-Pmel17-ls as the template. PMEL17-l-hemagglutinin (HA) was constructed by inserting the HA tag sequence between Gly\textsuperscript{173} and Thr\textsuperscript{174} of pCI-Pmel17-l as described above. LAMP-2-enhanced green fluorescent protein (EGFP) was constructed by inserting the PCR-amplified pCRII-LAMP-2 fragment into the Xhol-BamHI sites of pEGFP-N1 (Clontech). All constructs were sequence-verified and transfected into HeLa cells with Lipofectamine 2000 (Invitrogen) as described previously (15). Transfection efficiency was ~70–80% as assayed by monitoring fluorescence signals using the pEGFP-C3 vector.

**Antibodies and Reagents**—α-PEP13b and α-mSiN polyclonal antibodies were generated in rabbits against synthetic peptides corresponding to the C terminus of human PMEL17 and the N terminus of murine Silver, respectively (27). HMB45 (Dako Corp., Carpenteria, CA), HMB50 (NeoMarkers, Fremont, CA) and NK1/beteb (NeoMarkers) were used to detect PMEL17. Anti-LAMP-2 (Research Diagnostics, Inc., Flanders, NJ) and anti-green fluorescent protein (Abcam, Cambridge, MA) antibodies were also used.

**Immunofluorescence Microscopy**—Cells cultured on 2-well Lab-Tek Chamber Slides (Nalge Nunc International, Rochester, NY) were immunostained as described previously (15). The reactivities of Alexa Fluor 488, 594, and 647 (Molec-

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3 The abbreviations used are: LAMP, lysosome-associated membrane protein; TM, transmembrane domain; NTD, N-terminal domain; CTD, C-terminal domain; ER, endoplasmic reticulum; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; TGN, trans-Golgi network.

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ular Probes, Eugene, OR) were visualized as green, red, and blue signals, respectively. All preparations were examined with a Zeiss LSM 510 confocal microscope equipped with HeNe (543 and 633 nm), argon, and krypton laser sources.

Electron Microscopy—Electron microscopy was performed as described previously (15). Thin sections were examined using a Zeiss EM 912 Omega electron microscope.

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Protein Extraction and Immunoblotting—Cell extracts were prepared using the M-PER mammalian protein extraction reagent (Pierce), 1% Triton X-100 (Sigma), or 1% Nonidet P-40 (Calbiochem) in the presence of protease inhibitor mixture (Roche Applied Science). After centrifugation at 20,000 \( \times g \) for 30 min, the supernatants were harvested as soluble fractions. The protein concentration of each soluble fraction was measured using the BCA protein assay (Pierce). Pellets were washed once with the corresponding lysis buffer and then resuspended in 1X SDS-PAGE buffer as insoluble fractions. Equivalent amounts of soluble and insoluble fractions were used. Immunoblotting was performed as described previously (15).

Metabolic Labeling and Immunoprecipitation—HeLa cells were pulsed for 30 min with \([^{35}S]\)Met/Cys (GE Healthcare) and then chased for 4 h. Cells were lysed with lysis buffer containing 1% Triton X-100. Immunopurified samples were electrophoresed and visualized by fluorography as described previously (15).

RESULTS

**HMB45 Detects the Mo Form of PMEL17 by Immunoblotting**—Pigmented MNT-1 cells, unpigmented SK-MEL-28 cells, and HeLa cells transfected with empty vector, PMEL17-i, or PMEL17-is were analyzed by immunoblotting using HMB45 and \( \alpha\)PEP13h antibody. MNT-1 cells, SK-MEL-28 cells, and HeLa cells overexpressing PMEL17-i or PMEL17-is showed HMB45-positive ~30–50-kDa bands in common (Fig. 2, left panel), as reported previously (27, 29). PMEL17-i showed three distinct bands (or clusters of bands), one at ~100 kDa, another at ~50 kDa, and another at ~30 kDa (with a faint band at ~120 kDa). PMEL17-is also showed three distinct bands, but at ~80, ~45, and ~30 kDa (with a faint band ~110 kDa). Equivalents of those bands were also found in MNT-1 cells, although the reactivities for some of them were relatively lower. The difference between the PMEL17-i and PMEL17-is constructs is only a small 7-amino acid deletion in the RPT domain, which implies that the differences in HMB45 staining patterns is closely associated with the RPT domain. Only two fast
migrating and minor HMB45-reactive bands were detected in SK-MEL-28 cells, the fastest being somewhat larger than that seen in MNT-1 cells or in PMEL17-transfected HeLa cells. This might be due a variation in post-translational modifications, including glycosylation and/or sialylation, but is not simply due to the fact that SK-MEL-28 cells are unpigmented (31).

We also performed immunoblotting using anti-PEP13h antibody, which reacts specifically with the C terminus of PMEL17 (27). P1 or P1s (the short form of P1 derived from PMEL17-is or PMEL17-ls) and Mf3 were clearly identified in MNT-1 cells and in PMEL17-i- and PMEL17-is-transfected cells (Fig. 2, right panel). An unknown band, P75 (~75 kDa), was detected in MNT-1 cells (15); however, it was not detected in HeLa cells overexpressing any isoforms of PMEL17 (Fig. 2, right panel).

What are those high molecular mass bands (~100 or 80 kDa) that are recognized by HMB45 except in SK-MEL-28 cells? Similar bands were visible in some previous reports, but were not mentioned, probably because they were considered to be nonspecific (15, 27, 29, 32). Comparison of immunoblots using HMB45 or α-PEP13h antibody revealed bands at ~120 kDa in PMEL17-i and at ~110 kDa in PMEL17-is that represent P2 and P2s (the short form of P2 derived from PMEL17-is or PMEL17-ls), respectively. This raises the question at to why P1 was not detected by HMB45 in that extract. HMB45 requires sialylation to generate its epitope (27, 29, 30), sialylation being the terminal step of glycosylation. P1 then is the ER-modified form that is not detected by HMB45. Taken together, bands at ~120, 110, 100, and 80 kDa correspond to P2, P2s, Mα, and Mαs (the short form of Mα, which are derived from PMEL17-is or -ls), respectively, whereas the smaller ~30–60 kDa bands are derivatives of Mα or Mαs; thus, we tentatively name them MαC, MαC1, MαC2, and MαC3 (Fig. 2, left panel).

**HMB45 Recognizes the RPT Domain of PMEL17**—To identify the HMB45-reactive domain of PMEL17, we designed a series of 10 deletion mutants of PMEL17-i (ΔSIG, ΔNTD, ΔPKD, ΔGAP1, ΔRPT, ΔGAP2, ΔKRG, ΔGAP3, ΔTM, and ΔCTD) and then reacted with Alexa Fluor 488. Scale bar = 20 μm. B, HeLa cells transfected with mutants of PMEL17 (ΔSIG, ΔNTD, ΔPKD, ΔGAP1, ΔRPT, ΔGAP2, ΔKRG, ΔGAP3, ΔTM, ΔCTD, and ΔCS) were analyzed by immunoblotting using HMB45 (left panel) or α-PEP13h antibody (right panel). The numbers to the right indicate sizes in kilodaltons, and the specific bands as discussed under “Results” are indicated to the left. The single and double asterisks indicate specific bands detected by HMB45, also as discussed under “Results.”
with HMB45, and reactivity with ΔTM was greatly reduced. All other constructs reacted well, although HMB45-positive granules in ΔNTD, ΔPKD, ΔGAP1, and ΔGAP2 were located mainly in the perinuclear region rather than being dispersed in the cytoplasm.

The reactivities of these constructs were also analyzed by immunoblotting using HMB45 and α-PEP13h antibody (Fig. 3B). ΔSIG showed no reactivity with HMB45, and only a single band that migrated faster than P1 was detected by α-PEP13h antibody. Signal peptides classically function to target proteins to enter the ER; thus, it is reasonable that ΔSIG would not undergo glycosylation, would have a relatively smaller P1, and would not react with HMB45. According to a previous report that the P2-to-Mα/MB cleavage occurs in post-Golgi and prelysosomal compartments (16), it is also reasonable that Mβ was not detected in ΔSIG. These results suggest that SIG in PMEL17 is essential for entering the secretory pathway, as expected. ΔNTD showed two bands recognized by HMB45 at relatively lower intensities, a major one that was smaller than Mα and the other corresponding to MαC. However, the major bands (MαC1 and MαC3) were not detected by HMB45. P1 and P2 were identified in ΔNTD by α-PEP13h antibody staining, but their molecular masses were smaller than those of PMEL17-i. Mβ was also clearly detected in this mutant by α-PEP13h antibody. ΔPKD and ΔGAP1 had similar characteristics, showing faint single bands (Fig. 3B, * and **) that were not observed in any other constructs by HMB45. These bands were clearly located between P2 and Mα. Mβ was only faintly detectable in ΔPKD and ΔGAP1 by α-PEP13h antibody staining; however, P1 was clearly detected in both. This means that cleavage into Mα/Mβ is significantly hampered by deletion of the PKD or GAP1 domain.

However, ΔRPT showed the most striking feature in that HMB45 reactivity was completely lost, but α-PEP13h antibody reactivity with (truncated) P1 and Mβ was not affected. The reactivity of ΔGAP2 with HMB45 also showed only one slower migrating band compared with Mα; thus, the GAP2 domain might affect the processing of Mα. P1 was detected in ΔGAP2, but Mβ was not detected using α-PEP13h antibody because the furin-mediated cleavage site is located in the GAP2 domain, and thus, ΔGAP2 should not be able to produce Mβ because of its design. ΔKRG showed a significantly lower level of Mα detected by HMB45, although reactivity with MαC1 or MαC3 was not affected. Mβ was nearly undetectable, although P1 was clearly detected by α-PEP13h antibody. ΔGAP3 showed no difference in staining pattern from PMEL17-i, except for its truncated Mβ band detected by α-PEP13h antibody. ΔTM was nearly undetectable by immunoblotting using HMB45. Immunoblotting of ΔTM with α-PEP13h antibody showed P1 but not Mβ, which is similar to the pattern seen for ΔPKD and ΔGAP1.

Thus, the disruption of HMB45 reactivity seen for ΔPKD, ΔGAP1, and ΔTM indicates that these domains undergo glycosylation, but are not sufficiently sialylated to be recognized. Interestingly, none of them are significantly cleaved by proprotein convertases. Is that just a coincidence? Sialylation is performed by sialyltransferases, which require acidic pH optima (33), and furin proprotein convertases also require acidic conditions (34). One interpretation that would account for this is the failure to traffic to acidic compartments such as the trans-Golgi network (TGN). ΔCTD showed virtually the same immunoblotting pattern as PMEL17-i with HMB45, but was undetectable by α-PEP13h antibody, which was quite unexpected and is discussed in further detail below. The ΔCS mutation inhibited the production of Mα/Mβ; however, MαC1 and MαC3 remained unchanged.

N-terminal Domains Affect the Trafficking of PMEL17—We then examined whether deletion mutants of PMEL17 (excluding ΔSIG, ΔTM, or ΔRPT) are trafficked to late endosomes or lysosomes. We (15, 35) and others (12, 21) have previously reported that the trafficking of PMEL17 can be monitored by co-localization with LAMP-2 or LAMP-1, both of which localize in late endosomes, lysosomes, and melanosomes. We characterized the trafficking of mutant PMEL17 in HeLa cells by staining with HMB45 and by cotransfection of LAMP-2-EGFP. In PMEL17-i-transfected HeLa cells, HMB45 and LAMP-2 co-localized well (Fig. 4), and this also occurred in ΔKRG-, ΔGAP3-, and ΔCTD-transfected cells. However, in ΔNTD-, ΔPKD-, ΔGAP1-, or ΔGAP2-transfected cells, the number of HMB45-positive organelles was significantly less; they were localized only in the perinuclear region, and few co-localized with LAMP-2. Interestingly, in contrast to ΔPKD and ΔGAP1, ΔNTD and ΔGAP2 showed stronger HMB45 reactivity, and the Mβ fragment was clearly observed in ΔNTD. Both sialylation (required for HMB45 reactivity) and furin proprotein convertases (required for Mβ cleavage) require acidic pH optima (33, 34). This might account for the significant loss of HMB45 reactivity and the Mβ fragment detected by α-PEP13h antibody in ΔPKD and ΔGAP1 as described above. In contrast, ΔNTD and ΔGAP2 could reach the acidic environment, probably the TGN and later compartments. These results suggest that N-terminal domains (NTD, PKD, GAP1, and GAP2) are required for the correct trafficking of PMEL17 in these transfected cells. Moreover, the PKD and GAP1 domains seem to be required for entry into the early secretory pathway. In contrast to those luminal domain deletion mutants, C-terminal domains (KRG, GAP3, and CTD) are not required for the trafficking of PMEL17, at least in transfected HeLa cells. NMB shows high homology to PMEL17, and another study showed that a mutation of the putative dileucine motif in the cytoplasmic domain of QNR-71 (the quail homolog of NMB) clearly abrogated the trafficking of QNR-71 (36). PMEL17 also has di-leucine sorting signal motif at its C terminus. We further introduced di-alanine mutation at di-leucine motif of PMEL17 (LL662AA). As is the case with ΔCTD, LL662AA did not abrogate the trafficking of PMEL17 (Fig. 4) as it did in QNR-71.

The RPT Domain Is Required for the Proper Maturation of Fibrillogenesis—Previous reports by our group (14) and others (13) have shown that HMB45 specifically recognizes melanosomal matrix fibers. Moreover, HeLa cells can produce melanosomal matrix fiber-like structures when PMEL17 is overexpressed in the absence of other melanosomal proteins or melanosomes (12). When considered with the results of this study, it is clear that the RPT domain comprises the melanosomal matrix fiber itself. We analyzed what is observed in lysosomal organelles of HeLa cells overexpressing ΔRPT instead of PMEL17-i. We looked first at the intracellular trafficking of
ΔRPT. HeLa cells overexpressing PMEL17-i or ΔRPT were analyzed by immunocytochemistry using HMB50 (another antibody recognizing PMEL17) and anti-LAMP-2 antibody (Fig. 5A). PMEL17-i and ΔRPT co-localized similarly with LAMP-2 at high frequency, suggesting that ΔRPT is trafficked successfully to late endosomes or lysosomes, as is PMEL17-i. We then examined this at the ultrastructural level. Well developed parallel striated fibrillar structures similar to Stage II melanosomes were frequently observed in HeLa cells overexpressing PMEL17-i (Fig. 5B), as reported previously (12). In contrast, only occasional onion-like structures with electron-dense granules (termed lamellar bodies) were observed in HeLa cells overexpressing ΔRPT. Of all specimens examined, we found only one organelle with parallel striated fibrillar structure in HeLa cells transfected with ΔRPT (data not shown). From these results, we conclude that the RPT domain does not affect the trafficking of PMEL17; however, it plays a crucial role in the development of the striated fibrillar structure.

The RPT Domain Undergoes Variable Proteolytic Cleavage—We hypothesized previously that Mac is then processed to Mac1 and MacC (MacC2 for PMEL17-is or PMEL17-ls). The difference in HMB45 immunoreactivity between PMEL17-i and PMEL17-is suggests that this processing should occur in the RPT domain because PMEL17-is has a truncated RPT domain, and other domains of PMEL17-is are identical to those of PMEL17-i (Fig. 1A). The RPT domain consists of 10 imperfect repeats of 13 amino acids. We tried to identify which repeats correspond to each subdomain as detected by HMB45 and which repeats are identified by HMB45. To examine this, we designed a series of deletion mutants. RPTa–i has 9 of the 10 repeats (from a to i), RPTa–b has two repeats (a and b), RPTb–j has nine repeats (from b to j), and so on (Fig. 1A). Moreover, RPTsa–i has five repeats (a–d and i) and two truncated repeats (e and h) that are specific to PMEL17-is (or PMEL17-ls). We transfected those constructs into HeLa cells and performed immunoblotting using HMB45. The reactivity of HMB45 was lost in RPTa–b, RPTc–j, RPTd–j, and RPTe–j (Fig. 6A). This shows clearly that both repeats b and c are required for the reactivity of HMB45. These two repeats are also predicted to have potential O-glyco-
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A

Pmel17-i

\(\Delta\)RPT

HMB50

LAMP-2

Merged

B

Pmel17-i

\(\Delta\)RPT

FIGURE 5. HeLa cells overexpressing \(\Delta\)RPT analyzed immunocytochemically and ultrastructurally. A, HeLa cells overexpressing Pmel17-i or \(\Delta\)RPT were fixed and stained with antibodies. HMB50 and LAMP-2 were then reacted with Alexa Fluor 594 (red) and Alexa Fluor 488 (green), respectively. Scale bar = 20 \(\mu\)m. B, shown are the effects of the RPT domain on fibrillar structures. HeLa cells overexpressing Pmel17-i or \(\Delta\)RPT were fixed and observed by electron microscopy. Well developed fibrillar structures were seen in HeLa cells overexpressing Pmel17-i (left panel, arrow). Atypical lamellar bodies were occasionally seen in HeLa cells overexpressing \(\Delta\)RPT (right panel, dotted arrow). Scale bar = 200 nm.

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sylation sites (Fig. 1A). Repeats b and c do not have potential N-glycosylation sites. Moreover, digestion with peptide N-glycosidase F did not affect immunoreactivity with HMB45 (data not shown), suggesting that sialic acids recognized by HMB45 are located in repeats b and/or c. M\(\alpha\)c, M\(\alpha\)i, MoC, MoC1, and MoC3 migrated faster in RPTb–j than in PMEL17-i. This suggests that repeat a is involved in M\(\alpha\)c, MoC, MoC1, and MoC3. MoC was not always clearly detected in extracts of PMEL17-i (Figs. 2 and 6A). However, from the results obtained with other transfec-
tants, all repeats (from a to j) are involved in MoC, which proves that its C-terminal fragment is derived from M\(\alpha\)c (as shown schematically in Fig. 7).

Next, we tried to identify the C-terminal cleavage sites of MoC1, MoC2, and MoC3 (Fig. 6A). Similar to the case in PMEL17-i (data not shown), the \(\Delta\)CS mutations (Fig. 1A) did not affect MoC1, MoC2, or MoC3 (Figs. 3B and 6A). This suggests that the furin-mediated cleavage site does not affect MoC2 or MoC3 as detected by HMB45. MoC1 in PMEL17-i was detected in RPTa–h, but not in RPTa–g (Fig. 6A); thus, the cleavage must occur in repeat h in the RPT domain (Figs. 1A and 7). MoC2 in PMEL17-is was detected in RPTa–h, but not in RPTa–d (Fig. 6A), meaning that it is cleaved in repeat e or h in the RPT domain (Figs. 1A and 7). MoC3 was detected in RPTa–c, but not in RPTa–b (Fig. 6A); thus, it must be cleaved in repeat c in the RPT domain (Figs. 1A and 7). In PMEL17-is or PMEL17-Is (Fig. 6A), we could not identify MoCs (the short form of MoC; that derived from PMEL17-is or -Is, which might be due to the fact that its size is too similar compared with MoC2 and/or that MoCs is a transient form. Taken together, these results suggest that the RPT domain undergoes variable proteolytic cleavage.

MoN has been reported to have the same molecular mass as M\(\beta\), which is \(\sim 26\) kDa (27). However, in this study, M\(\alpha\) is \(\sim 100\) kDa and MoC is \(\sim 60\) kDa, so MoN is estimated to be \(\sim 40\) kDa (Figs. 1B and 3A). Why should this discrepancy occur? MoN was detected by pulse-chase labeling and immunoprecipitation with an antibody that recognizes the N terminus of PMEL17 (\(\alpha\)-mSiN antibody) (27). We re-examined this question using HeLa cells overexpressing PMEL17-i or \(\Delta\)GA3 (the GAP3 domain is part of M\(\beta\) that was radiolabeled and then chased for 4 h. Cells were solubilized and immunoprecipitated with \(\alpha\)-PEP13h or \(\alpha\)-mSiN antibody and then electrophoresed and observed by fluorography (Fig. 6B). \(\alpha\)-PEP13h antibody recognized P1 and M\(\beta\) in PMEL17-i, as did \(\alpha\)-mSiN antibody. \(\alpha\)-PEP13h antibody also reacted with P1 and with truncated M\(\beta\) in \(\Delta\)GA3; and interestingly, \(\alpha\)-mSiN antibody also recognized that same truncated M\(\beta\) band. Thus, what was reported previously as MoN is the same as M\(\beta\). Even after the production of M\(\beta\), M\(\alpha\) and M\(\beta\) remain linked, probably via disulfide bonds (12). \(\alpha\)-mSiN antibody would be expected to immuno-react with P1, P2, and the MoN\# complex (Fig. 1B) under the previously reported conditions of immuno-
precipitation (27). However, the subsequent electrophoresis was performed under denaturing conditions, in which the MoN\# complex would be dissociated (12, 27).

So the challenge is how can we detect MoN? To do this, we artificially inserted an HA tag into the NTD of PMEL17-i between the potential third N-glycosylation site and the PKD domain (PMEL17-i-HA). HeLa cells were transfected with PMEL17-i-HA and then digested with or without peptide N-glycosidase F because MoN contains N-glycosylation sites (Fig. 1B). We then analyzed the digests by immunoblotting with an antibody that recog-
nizes the N terminus of PMEL17-i, as did \(\alpha\)-mSiN antibody. \(\alpha\)-PEP13h antibody also reacted with P1 and with truncated M\(\beta\) in \(\Delta\)GA3; and interestingly, \(\alpha\)-mSiN antibody also recognized that same truncated M\(\beta\) band. Thus, what was reported previously as MoN is the same as M\(\beta\). Even after the production of M\(\beta\), M\(\alpha\) and M\(\beta\) remain linked, probably via disulfide bonds (12). \(\alpha\)-mSiN antibody would be expected to immuno-react with P1, P2, and the MoN\# complex (Fig. 1B) under the previously reported conditions of immuno-
precipitation (27). However, the subsequent electrophoresis was performed under denaturing conditions, in which the MoN\# complex would be dissociated (12, 27).
One interesting report showed that Mα is buried in the Triton X-100-insoluble fraction (16). We used M-PER cell lysis buffer because HMB45 reactivity is stronger with this buffer than following extraction with Triton X-100 or Nonidet P-40 (27). We prepared soluble and insoluble fractions of MNT-1 cells solubilized with M-PER-, 1% Triton X-100-, or 1% Nonidet P-40-based lysis buffers and then immunoblotted these extracts using HMB45 or α-PEP13h antibody (Fig. 6D). In the soluble fractions, the reactivity of HMB45 was strongest with M-PER solubilization, as reported previously (27). Interestingly, in the insoluble fractions, which were not examined in the previous study (27), HMB45 reactivity was stronger following extraction with Triton X-100 or Nonidet P-40 and was weaker following extraction with M-PER reagent. In contrast, α-PEP13h antibody-detectable forms of PMEL17 (P2, P1, P1s, and Mβ) were found mainly in the soluble fractions of all lysis buffers (Fig. 6D). From these results, it can be concluded that Mα and Mαs were highly enriched in the Triton X-100- and Nonidet P-40-insoluble fractions. Moreover, their derivatives (MαC, MαC3, MαC2, and MαC1) were also enriched in the Triton X-100- and Nonidet P-40-insoluble fractions. The exact composition of M-PER reagent has not been released, but HMB45-reactive materials are more efficiently solubilized by M-PER reagent.

Finally, we tried to clarify the N-terminal cleavage site(s) of Mα and Mαs, including MαC1, MαC2, and MαC3. According to the immunoblotting of ΔNTD using HMB45, MαC was detected (Fig. 3B, left panel), showing that the NTD is not involved. Immunoblotting of RPTb–j (deletion of repeat a in the RPT domain) using HMB45 showed faster migration of MαC1 and MαC2 (Fig. 6A), so the RPT domain is also not involved. HMB45 did not detect MαC in ΔPKD or ΔGAP1 (Fig. 3B, left panel), although the levels of Mβ were very low in these extracts, and perhaps it did not undergo cleavage. Taken together, the N-terminal cleavage site(s) of PMEL17 should be in ΔPKD and/or in ΔGAP1, but cannot be further identified from these results. Thus, it is clear that Mα is processed into MαN (~40 kDa) and MαC (~60 kDa), after which MαC is further processed into MαC1 or MαC3 (MαC2 in the case of PMEL17-is or PMEL17-ls).

**DISCUSSION**

In this study, we have demonstrated that HMB45 reacts with the RPT domain of PMEL17. HMB45 was initially developed for melanoma detection, but was later shown to react with PMEL17 and melanosomal matrix fibers (12–14). PMEL17 can produce fibrillar structures even following expression by transfection in non-melanocytic cells (12). Combining previously published results with those of this study, independent lines of evidence can now be linked at the subdomain
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The formation of the fibrillar matrix seen in Stage II melanosomes (14, 27). This route is supported by the fact that subcellular fractions of human melanoma cells enriched in Stage II melanosomes contain P1, but not P2 (14). Proteomic analysis of those fractions revealed ER-resident proteins (35). The endoglycosidase H-sensitive form of PMEL17 (P1) was detected, but the largely endoglycosidase H-resistant form (P2) could not be detected by immunoblotting of melanoma cell lysates (27). Another proposed sorting route is that PMEL17 is trafficked from the TGN to early endosomes and Stage I melanosomes and then finally to Stage II melanosomes (12). This is supported by the fact that P2 and Mo are largely endoglycosidase H-resistant, which means that N-acetylglucosaminyltransferase in the medial-Golgi is involved (12, 25). In a recent study (28), we showed that both trafficking pathways are in fact involved in the sorting of PMEL17. Both the major (P1) and minor (P2) forms of PMEL17 are delivered to early melanosome, but via distinct sorting pathways. In this present study, we have demonstrated that it is only the minor late Golgi-modified form (P2) that is critical to the formation of fibrils in melanosome. What function(s) the major ER-modified form of PMEL17 (P1) may play remains open.

This study has also shown that the HMB45-reactive RPT domain of PMEL17 is O-glycosylated with sialic acid modifications. Indeed, sialic acid modifications for N-glycans occur in the trans-Golgi or TGN (31). However, sialyltransferases responsible for the sialylation of O-glycans are located throughout the entire Golgi stack, so one cannot conclude from those results alone whether proteins have gone through the late Golgi (41, 42). Interestingly, Mo detected by HMB45 was largely endoglycosidase H-resistant and peptide N-glycosidase F-sensitive (data not shown), suggesting that the HMB45-reactive form of PMEL17 has been modified in the late Golgi. P2 and Mo are transient forms and are usually difficult to detect, especially PMEL17 expressed endogenously in melanocytic cells (Figs. 3A and 4A). Moreover, Mo is buried in the detergent-insoluble fraction, and its molecular mass is quite similar to that of P1 (Figs. 3A and 4A) (16). Thus, failure to detect P2 or Mo does not directly suggest that only P1 (but not P2) will be trafficked to melanosome. Mo and its derivatives can be clearly identified in M-PER reagent-soluble fractions using the sialylation-dependent antibody HMB45. The specificity of HMB45 allows it to recognize only Mo and P2, whereas the specificity of α-PEP13h antibody allows it to recognize only P1 and P2; thus, Mo and P1 can be readily distinguished with those two reagents. Derivatives of Mo (such as MαC1 and MαC3) that are detected by HMB45 indicate the existence of Mo and P2. It is also clear that HMB45 recognizes the fibrillar matrix in Stage II melanosomes at the ultrastructural level (12–14). Taken together, the fragment of PMEL17 found in Stage II melanosomes recognized by HMB45 is late Golgi-modified and must be trafficked from the TGN.

Is the proposal that P1 is trafficked from the ER or cis-Golgi to early melanosome correct? For a long time, it has been believed that melanosomes mature from Stages I to IV (11). When considered with other published studies, our results may raise the question, “Do Stage I melanosomes really mature to Stage II melanosomes?” The difference between Stage I and II
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melanosomes depends completely on the presence of the fibrillar structure (11). We reported recently that MART-1-negative but PMEL17-positive melanoma cells produce Stage I (but not Stage II) melanosomes (15). P1 was detected in subcellular fractions enriched in Stage I melanosomes, but HMB45 was negative in all subcellular fractions as well as in whole cell lysates. This suggests that P1, which is the major form of PMEL17, can be trafficked to Stage I melanosomes, but is not required for the generation of fibrils involved in the maturation to Stage II melanosomes. In contrast, the minor late Golgi-modified form of PMEL17 (P2) is trafficked from the TGN to Stage II melanosomes. From these points of view, both hypotheses for the trafficking of PMEL17 are compatible and convincing. PMEL17 is also secreted, a phenomenon not seen for TYR (tyrosinase), TYRP1 (tyrosinase-related protein 1), or dopachrome tautomerase in normal melanocytes (21). Thus, it is reasonable that multiple forms of PMEL17 might take different trafficking routes, although careful investigation is required to identify them (28).

By clarifying the characteristics of HMB45 recognition of PMEL17 and its fragments, our study has shed light on the trafficking as well as the processing of PMEL17 linked to early melanogenesis. Is late Golgi-modified PMEL17 sufficient to produce Stage II melanosomes? In this study, we focused only on PMEL17 and HMB45 using transfection of non-melanocytic cells. In recent studies, mice harboring mutations in pigment-related genes other than PMEL17 produced fibrillar structures; however, the numbers were few, and/or the shapes were not ellipsoidal (43, 44). Such factors should be considered for a better understanding of early melanogenesis.

In this study, we have shown that Mα is processed to MαN and MαC and that MαC is then further processed because of the variable proteolytic cleavage of the RPT domain. Where does such processing occur? Processing of Mα was observed for ΔNTD, which was not trafficked to LAMP-2-positive organelles (Figs. 3B and 6). The cleavage of P2 to Mα/MB has been reported to occur in post-Golgi and prelysosomal compartments (16). This suggests that the processing of Mα to MαC/MαN might occur in post-Golgi and/or prelysosomal compartments. However, such processing might occur independently because ΔPKD and ΔGAP1 showed some cleavage of P2 to Mα/MB, but did not show any cleavage of Mα to MαC/MαN (Fig. 3B). MαC1 and MαC3 were observed only in PMEL17-i-, ΔKRG-, ΔGAP3-, and ΔCTD-transfected cells, and all of them were efficiently trafficked to late endosomes or lysosomes (Figs. 3B and 6). ΔNTD was localized mainly in the perinuclear area, but could produce Mβ and MαC (Figs. 3A and 6). Taken together, the variable proteolytic cleavage of the RPT domain probably occurs in late endosomes or lysosomes, in other words, in melanosomes. Which enzymes are involved is another interesting issue, and future work will attempt to identify them.

The function of the KRG domain in PMEL17 is currently unknown. Mβ was undetectable in ΔKRG-transfected cells by α-PEP13h antibody (Fig. 3B, right panel). Accordingly, Mα detected in ΔKRG-transfected cells by HMB45 was significantly weaker than that detected in ΔGAP3-, ΔCTD-, or PMEL17-i-transfected cells (Fig. 3B, left panel). Interestingly, ΔKRG trafficked correctly to LAMP-2-positive organelles (Fig. 4). Recently, the kringle-2 domain of tissue plasminogen activator was shown to be necessary for the cleavage of platelet-derived growth factor CC at its tribasic processing site, 231RKSR (45). Moreover, the kringle-2 domain of tissue plasminogen activator directly binds platelet-derived growth factor CC. The enzyme that cleaves P2 into Mα/MB has not yet been specifically identified (16). However, it is possible that the KRG domain is necessary for efficient processing by that enzyme.

An unexpected and interesting result was found for ΔCTD. ΔCTD showed no detectable disruption in the processing or trafficking of PMEL17 in transfected cells (Figs. 3B and 6). Many melanosomal proteins have putative sorting signals in their cytoplasmic domains (46). PMEL17 has a putative dileucine-based signal (ENSSL) in its CTD. Equivalent dileucine-based sorting signals are found in TYRP1, TYR, and QNR-71 (the quail homolog of NMB), and mutations of those signals cause misrouting of those proteins (36, 47–49). Surprisingly, this may not hold true for PMEL17 because deletion of the CTD or mutation of the dileucine motif in PMEL17 did not disrupt sorting to late endosomes or lysosomes in transfected non-melanocytic cells. Whether the CTD plays a role in the sorting of PMEL17 in melanocytic cells is an important question to resolve, as is the identification of the precise signal(s) involved in the luminal region. The sorting determinants found in luminal domains of PMEL17 (such as NTD, PKD, GAP1, and GAP2) seem to be critical to the regulation of PMEL17 trafficking. This might account for the distinct nature of PMEL17 trafficking in melanocytes, which is different from the sorting of other melanosomal proteins such as TYR, TYRP1, and dopachrome tautomerase (14, 28). The PKD and GAP1 domains seem to be required for earlier events in the secretory pathway, whereas the NTD and GAP2 domain seem to be required for later events. Probably, they are involved in distinct sorting mechanisms. Among those domains, the PKD domain is the only conserved domain, and it might be involved in protein-protein and protein-carbohydrate interactions (50), although the function of the PKD domain is still unresolved at this time. This is a novel finding and a quite intriguing issue and will require further study. Future investigation will be directed to further identify sorting components that might utilize the luminal domains of PMEL17.

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Addendum—After submission of this manuscript, two independent studies reported the presence of multiple sorting signals for PMEL17 in the N-terminal/luminal and C-terminal domains (51, 52).
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