Sialylated Core 1 O-Glycans Influence the Sorting of Pmel17/gp100 and Determine Its Capacity to Form Fibrils

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Pmel17 is a melanocyte/melanoma-specific protein that is essential for the maturation of melanosomes to form mature, fibrillar, and pigmented organelles. Recently, we reported that the less glycosylated form of Pmel17 (termed iPmel17) is sorted via the plasma membrane in a manner distinct from mature Pmel17 (termed mPmel17), which is sorted directly to melanosomes. To clarify the mechanism(s) underlying the distinct processing and sorting of Pmel17, we generated a highly specific antibody (termed αPEP25h) against an epitope within the Pmel17 repeat domain of Pmel17 that is sensitive to changes in O-glycosylation. αPEP25h recognizes only iPmel17 and allows analysis of the processing and sorting of iPmel17 when compared with αPEP13h, an antibody that recognizes both iPmel17 and mPmel17. Our novel findings using αPEP25h demonstrate that iPmel17 differs from mPmel17 not only in its sensitivity to endoglycosidase H, but also in the content of core 1 O-glycans modified with sialic acid. This evidence reveals that iPmel17 is glycospayed differently in the Golgi and that it is sorted through the secretory pathway. Analysis of Pmel17 processing in glycosylation-deficient mutant cells reveals that Pmel17 lacking the correct addition of sialic acid and galactose loses the ability to form fibrils. Furthermore, we show that addition of sialic acid affects the stability and sorting of Pmel17 and reduces pigmentation. Alterations in sialyltransferase activity and substrates differ between normal and transformed melanocytes and may represent a critical change during malignant transformation.

The human PMEL17 gene encodes two type I integral membrane proteins that are translated from alternatively spliced mRNAs. Those two proteins, known as Pmel17 and gp100, differ as to whether they include a 7-amino acid motif in the membrane proximal region of the luminal domain (1–3). Pmel17 is critical to the formation of an internal fibrillar matrix within stage II melanosomes that is essential to the maturation of these organelle and the subsequent synthesis and deposition of melanin within it. Pmel17 is synthesized as a 68.6-kDa protein, as deduced from the cloned cDNA (2). It is rapidly and quantitatively glycosylated to an "immature" ~95-kDa form (termed iPmel17), but only a limited amount is further processed to a "mature" ~115-kDa form (termed mPmel17), due at least in part to N-glycosylation at five possible sites (4). mPmel17 can then be cleaved by a furin-like proprotein convertase that generates two fragments, one small fragment (cPmel17, ~26 kDa) containing the transmembrane (TM) and the C-terminal domains, and the other larger fragment contains the N-terminal region (5). Those fragments can remain bound to each other by S–S bonds (5, 6). However, the iPmel17 formed remains fully endoglycosidase H (EndoH)-sensitive (4) and is significantly more stable over time than either of the other forms of Pmel17 (mPmel17 and cPmel17) (5).

N-Linked core glycosylation begins in the ER and is essential for the function, stability, folding, intracellular transport, and secretion of glycoproteins (for review see Ref. 7). Upon arrival in the cis-Golgi, the initial mannosese-rich core chains are trimmed by mannosidases I and II to continue N-glycosylation and O-linked glycosylation starts but may occur at multiple sites within the Golgi (8). In the medial- and trans-Golgi, the formation of complex type glycans ensues, which is terminated by the addition of sialic acid or other sugars. Sialyltransferases are responsible for the addition of sialic acid. Changes in the expression and enzyme activity of ST3Gal I and ST6Gal II, which catalyze the addition of sialic acid in positions α2,3 or α2,6, respectively, are associated with alterations in the length and structure of glycoproteins in several tumors and have been associated with malignant potential (9, 10). In melanoma cells, altered sialylation of surface glycoconjugates has been associ-
ated with a highly metastatic phenotype (11–13). Despite the importance of sialic acid addition, no study has reported sialylation transferase activity or its role in the sorting of melanosome-specific proteins in various types of melanocytic cells.

N-Glycans on Pmel17 contains a mixture of complex, hybrid, and mannose-rich carbohydrate chains that distinguish its secreted and cytoplasmic forms (3). In contrast, no information for the O-glycan content of Pmel17 is available. Recently, we reported that a glycoform of Pmel17 (iPmel17) is sorted via the plasma membrane in a manner distinct from mPmel17, which is sorted directly to melanosomes (14). These facts suggest that Pmel17 can be glycosylated differently and that those forms may have distinct functions. The phenomenon that any given glycosylation site on a given protein synthesized by a particular cell type can have a range of variations in the precise glycan structure is known as microheterogeneity (15). To date, there have been no reports that address this issue regarding melanosome-specific proteins.

The putative function(s) of iPmel17, which is the major form of Pmel17 seen in pigmented cells, is a matter of conjecture and controversy at this point (16, 17). The rapid processing of Pmel17 and the lack of in depth glycosylation studies have been impediments to elucidating mechanisms involved in the complex processing, trafficking, and functions of the various forms of Pmel17. To develop another tool to resolve some of these issues, we designed a highly specific antibody against a peptide sequence in the core region of Pmel17. That antibody, termed αPEP25h, specifically recognizes iPmel17 and has allowed us to characterize the different processing events that generate this important fragment. Reactivity with αPEP25h can be blocked by glycosylation at residues adjacent to its epitope, allowing us to study iPmel17 processing and sorting compared with mPmel17 that is recognized by αPEP13h. We demonstrate for the first time that iPmel17 differs from mPmel17 by having different sialylated core 1 O-glycans. Furthermore, we show that Pmel17 deficient in sialic acid and galactose loses the ability to form fibrils, and thus the addition of sialic acid at the α2,3-position is an important determinant of Pmel17 sorting through the secretory pathway.

**MATERIALS AND METHODS**

*Cell Cultures, Human Tissue Samples, and Skin Biopsies—* Pigmented (MNT-1) human malignant melanoma cells and HeLa cells were cultured as described previously (18, 19, 20). CHO cells as well as Lec2 and Lec8 mutant CHO cells were cultured as described previously (21). Cell extracts from neuroblastoma cell lines (SKNAS and SKNSH) were gifts from Dr. Maria Tsokos, NCI, National Institutes of Health. The human melanocyte cell line M253 was cultured in 154 Medium supplemented with growth factors as described by Cascade Biologics (Portland, OR). Paraffin-embedded samples from human kidney, uterus, and lung were obtained from the tissue bank in Philadelphia. Shave biopsies, 4 mm in diameter, were taken before and 1 day after a 1 minute erythema dose of UV radiation, as described previously (22).

*Antibodies, Peptide Synthesis, and Immunoaffinity Purification—* αPEP13h recognizes the C terminus of human Pmel17 (18). The αPEP25h polyclonal antibody was raised against a keyhole limpet hemocyanin-conjugated synthetic peptide (keyhole limpet hemocyanin-CTPEATGMPTEAV-SIVVLSGT-CO2H) by immunization in rabbits as reported previously (23, 24). Peptides used for affinity purification were synthesized by the solid phase method with 9-fluorenylmethoxy-carbonyl chemistry using a 431A peptide synthesizer (Applied Biosystems, Foster City, CA). Each peptide was purified by high pressure liquid chromatography on a Vydac C-4 column with 0.05% trifluoroacetic acid/water/acetonitrile. The mass of each peptide was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Micromass, Beverly, MA). Sera from immunized rabbits were affinity-purified using each peptide coupled with SulfoLink (Pierce). The specificity of each antibody was confirmed by enzyme-linked immunosorbent assay using synthetic peptides.

Monoclonal antibodies used include HMB-45 against Pmel17 (DAKO, Carpinteria, CA), HMB-50 and MART1 (NeoMarkers, UK), and anti-V5 (Invitrogen). Other monoclonal antibodies used as intracellular organelle markers are as follows: BIP (ER), Vti1b (Golgi), clathrin, and adaptin α (AP2) all from BD Transduction Laboratories.

*Mass Spectrometry—* Immunoprecipitated samples were separated on Tris-glycine gels and stained with the colloidal blue staining kit (Invitrogen) according to the manufacturer’s instructions. Retrieved protein bands were reduced, carbamidomethylated, and in-gel digested with trypsin using the Montage In-Gel Digest_25 kit (Millipore, Billerica, MA) according to the manufacturer’s directions. Peptide separation was performed at 300 nl/min and was coupled to on-line analysis by nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry on an LTQ ion trap mass spectrometer (ThermoElectron, San Jose, CA) equipped with a nanospray ion source. From the eluted peptides, 2 μl were loaded onto a 0.075 × 50 mm PicoFrit BioBasic C18 packed tip column (New Objective, Woburn, MA) using the Paradigm MS4 MDLC (Michrom Bioresources, Auburn, CA). Elution of peptides into the mass spectrometer was performed with a linear gradient from 95% mobile phase A (5% acetonitrile, 0.5% acetic acid, 94.5% water) to 65% mobile phase B (5% water, 0.5% acetic acid, 94.95% acetonitrile) in 45 min and then to 95% mobile phase B in 5 min. Tandem mass spectra were searched against the human NCBI data base using TurboSEQUENT in BioWorks version 3.2 (ThermoElectron, San Jose, CA).

*Fluorescence and Electron Microscopy—* For dual immunofluorescence staining, cells were grown in 2-well chamber slides (Nalgene, Naperville, IL) and fixed with 4% paraformaldehyde for 15 min at 4 °C. Cells were then incubated with primary antibodies overnight at 4 °C, followed by incubation with Texas Red anti-rabbit or fluorescein isothiocyanate streptavidin for polyclonal and monoclonal antibodies, respectively (25). Human skin specimens embedded in paraffin were processed as described previously (26). Fluorescence signals were classified according to whether they showed green, red, or yellow fluorescence, the latter being indicative of colocalization of the red and green fluorescence signals. Images were obtained using an LSM 510 confocal microscope (Zeiss, Jena, Germany). Analysis and quantification of the colocalization signal were evaluated under equal magnification, laser intensity, and saturation in the same.
preparations using Zeiss colocalization software. For each staining condition, 10 optical fields were observed, and representative images from two separate experiments are shown in the figures. For electron microscopy and immuno-electron microscopy, we used a previously published method without modifications (20).

Expression Vector Cloning and Transfection of the MART1 and Pmel17 Genes—The MART1 vector was a gift from Dr. Toshihiko Hoashi (Tokyo, Japan). DNA oligonucleotides for gp100 PCR were synthesized and purified by Operon. The forward primer is 5′-GGG ATG CAT CTG GTG CTA AAA AGA TGC CTT CCT C-3′, and the reverse primers are 5′-CAC CAG CCT TAA GTT GGC TGT ACC ATC CAG-3′ and 5′-GAC CTG CTG CCC ACT GAG GAG GGG GCT ATT-3′. These primers were used to construct expression vectors for the full-length gp100 cDNA (pcDNA5/Pmel17fl-V5) and its truncated form (pcDNA5/Pmel17Δ-V5) that contains the N-terminal 468 amino acids. The DNA fragments were amplified from a gp100-containing plasmid (clone IOH4070 open reading frame; Invitrogen) by 35 cycles of PCR as described previously (27). The PCR fragments were subcloned into the pcDNA5/FRT-V5-His6 vector (Invitrogen) confirmed by NheI and BamHI digestion and by DNA sequencing (the DNA Sequencing Facility, NCI, Bethesda). The plasmids were prepared using a Qiagen plasmid extraction kit. Transient transfection of full-length Pmel17 (FL-Pmel17), cleaved N-terminal fragment (cNTF-Pmel17), and a mock FRT control vector all dual tagged with V5 and His into MNT-1 cells, CHO, Lec2, and Lec8 cells was achieved by employing Lipofectamine 2000 (Invitrogen) or by electroporation using the Nucleofector kits and equipment (AMAXA, Gaithersburg, MD) according to the manufacturer’s instructions.

Immunoblotting and Sample Preparation—For immunoblotting, cell extracts or isolated fractions were prepared as described previously (20). Briefly, cell extracts were mixed with sample buffer (2×) (Invitrogen) supplemented with 2-mercaptoethanol and heated for 5 min at 100 °C. Samples were then separated on 10, 8–16%, or 4–20% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Bio-Rad) for 1 h at 100 V. The membranes were blocked in 5% nonfat dry milk in 1× Tris-buffered saline (TBS) for 1 h and then incubated with primary antibodies overnight at 4 °C. The membranes were washed for 15 min in TBS-T and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Primary antibodies were detected using ECL plus system (Amersham Biosciences), according to the supplier’s instructions.

Sialyltransferase Assays—Assays were performed as described previously (28). Briefly, enzyme activity was measured at 37 °C for 4 h in 50 μl of reaction mixture comprising 0.1 M cacodylate buffer, pH 6.2, 10 mM MnCl2, 0.2% Triton CF-54, 50 μM CMP-[14C]Neu5Ac (1.85 kBq) (Amersham Biosciences), with 23 μl of the enzyme source and 1 mg/ml of fetuin, asialofetuin, orosomucoid, asialo-orosomucoid, bovine submaxillary mucin (BSM), or asialo-BSM (Sigma) as acceptor substrates. The reactions were stopped by addition of 1 ml of H2O. Proteins were precipitated in 1 ml of 5% phosphotungstic acid in 2 N HCl and filtered on GF/A glass microfiber filters (Whatman). The radioactive material present on each filter was counted by liquid scintillation. The rate of this reaction was linear with time at least for 8 h. All reactions were performed in triplicate.

Quantitative Real Time PCRs—Total cytoplasmic RNA was isolated from cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the supplier’s instructions. Reverse transcription was performed using 200 ng of cytoplasmic RNA as reported previously (14). All amplified products were sequence-verified. Quantitative real time PCRs were performed using the Opticon analysis system (MJ Research Inc., Waltham, MA) and a hot-start PCR that contained the double-stranded specific DNA-binding dye SYBR Green 1 (Sigma). The following primers and their product sizes were used: β-actin, 5′-CCCTCCATCGTCCACCGCAA-ATGCTTC and 3′-GACTGCTGTGACCTCCAGTTC- CAG, 204 bp; ST3Gal I, 5′-TTCTCACCCTCCCTTCCTG- AACTAC and 3′-TCTTCTCCAGCAGATGGTGCACAC- TCC, 346 bp; and St6GalNAc II, 5′-GGAAATGTCGGTGGA GTGTTCAGCAAG and 3′-AAGCAACTAACCCTATC- AAGTGCAGACACTTC, 356 bp. After 5 min at 95 °C, 40 cycles were performed as follows: 15 s denaturation at 94 °C, 30 s annealing at 60 °C, 30 s extension at 72 °C, and fluorescence detection at 78 °C. A melting curve fluorescence analysis was performed on each sample once the amplification cycles were completed to verify that a single product had been amplified. The Ct is defined as the point when the amplification starts the exponential phase (29). The fold difference was calculated by subtracting the Ct of the test sample from the Ct of actin to give ΔCt, and then fold difference = 2−ΔCt.

Reagents and Enzymes—Cells were treated with the following reagents (all from Calbiochem): 10 μM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid overnight (30); 50 μM deoxyarn-A-Val-Lys-Arm-Arg-Val-lys-Arg-CMK for 3 h (31), 50 μM brefeldin A (20), and 10 mM benzylxoxy-carbonyl-Leu-Leu-Leu-al (MG132) (32).

N-Glycan chains were analyzed by digestion with EndoH or PNGaseF according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA). Modifications to glycans were analyzed with α-2,3,6,8,9-neuraminidase, endo-α-N-acetylgalactosaminidase (O-glycans), β1,4-galactosidase, and β-N- acetylgalactosaminidase (Calbiochem), according to the manufacturer’s instructions. Enzyme digestion with EndoH (New England Biolabs) for 35S-labeled samples was performed as described previously (20). Briefly, Pmel17 samples were eluted from the protein A-Sepharose beads with denaturing buffer after heating for 5 min at 100 °C. Labeled samples were digested in reaction buffer with 500 units of EndoH for 18 h at 37 °C, separated by SDS-PAGE, and further analyzed by autoradiography.

Metabolic Labeling—Metabolic labeling and immunoprecipitation were performed as described previously (20). Briefly, cells were cultured in 10-cm dishes until 80% confluent. Cells were preincubated in methionine-free medium for 30 min at 37 °C and were then labeled for 30 min with 0.5 μCi of [35S]Met/Cys (Redivue Pro Mix; Amersham Biosciences) and chased in methionine-plus medium for the times indicated in the text. After harvesting, cells were washed and incubated in lysis buffer overnight (33). Samples were pre-cleared with normal rabbit serum and protein G-Sepharose 4 fast flow beads
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(Amersham Biosciences) for 2 h at 4 °C. The supernatants were collected and immunoprecipitated with either αPEP13h or αPEP25h for 2 h at 4 °C, and immunocomplexes were then separated with protein G- Sepharose beads (Amersham Biosciences) for 2 h at 4 °C. For double immunoprecipitation, we recovered these supernatants and immunoprecipitated them again with either αPEP13h or αPEP25h for 2 h at 4 °C and then recovered the immunocomplexes again with protein-G beads and continued with the protocol. After washing, the final pellets were suspended in SDS sample buffer (Invitrogen) with 2-mercaptoethanol (Sigma), heated for 5 min at 100 °C, and separated on 10 or 8–16% Tris-glycine gels (Invitrogen). Control samples were immunoprecipitated with normal rabbit serum and were processed in parallel.

Lectin Staining—Lectin blot analysis using digoxigenin-labeled lectins (Roche Applied Science) was performed according to the manufacturer’s instructions.

Melanin Content Assay—Melanin content was determined as described previously (34). In brief, cell pellets were dissolved in 100 μl of 1 N NaOH, and melanin concentrations were quantitated by absorbance at 405 nm using a standard curve generated from synthetic melanin (Sigma). For each condition, the melanin content is expressed as nanograms of melanin divided by total protein concentration in micrograms. Values are then reported as a percentage of those obtained in controls. Each experiment was repeated at least two times.

RESULTS

αPEP25h Specifically Detects iPmel17—The antibody αPEP25h was designed against residues 393–414 (Fig. 1A) in the core region of Pmel17 (35, 36). αPEP25h would be expected to recognize only the two major splice forms of Pmel17 and not a recently reported minor third splice variant (37). To assess the sensitivity and specificity of αPEP25h, we affinity-purified it from rabbit antiserum and compared its reactivity patterns against the well characterized αPEP13h (which recognizes the C terminus of Pmel17). An enzyme-linked immunosorbent assay showed that both purified antibodies, αPEP25h and αPEP13h, have similar binding curves and high specificities for their immunizing peptides (supplemental Fig. 1).

To analyze the specificity of αPEP25h for Pmel17, we used MNT-1 melanoma cells, which remain quite differentiated and nonmelanocytic cell lines (SKNAS and SKNSH neuroblastoma cells, HeLa cells, and human primary fibroblasts). αPEP13h identified Pmel17 only in melanocytic cells as expected and also detected minor bands of mPmel17 and cPmel17, along with an even smaller fragment (~10 kDa) in MNT-1 cells (Fig. 1C, arrow). In contrast, αPEP25h specifically recognized Pmel17 only as a single major band (~95 kDa) and only in melanocytic cells. As detected by αPEP25h and by αPEP13h, the iPmel17 band was sensitive to digestion with EndoH (which removes high mannose/hybrid \(N\)-glycans) and with PNGaseF (which removes all \(N\)-glycans) (Fig. 1D).

The band recognized by αPEP25h is identical in size to iPmel17 as detected by αPEP13h. To determine whether they are in fact the same, we performed sequential immunoprecipitation using the \(^{35}\)S-labeled samples after a 45-min chase, a time when all Pmel17 bands were observed (Fig. 1E). Fig. 1E, lane 1, shows bands immunoprecipitated with αPEP13h alone, but if the sample had been immunodepleted by αPEP25h, little of the ~95-kDa band remained and could be immunoprecipitated by αPEP13h (lane 2). Conversely, immunodepletion with αPEP13h first followed by immunoprecipitation with αPEP25h resulted in an 85% depletion of the band recognized by αPEP25h (Fig. 1E, lane 4). Bands immunoprecipitated by preimmune sera are shown in Fig. 1E, lanes 5 and 6, as controls for nonspecific binding. We conclude that the ~95-kDa bands recognized by αPEP13h and by αPEP25h are in fact the same protein, iPmel17. To further confirm this, we immunoprecipitated Pmel17 with αPEP25h and with αPEP13h from MNT-1 cell lysates and analyzed the major band detected by each antibody using mass spectrometry. Several Pmel17 tryptic peptides (Table 1), each showing unique tandem mass spectra (supplemental Fig. 2), were identified in those bands with >95% confidence. The sum of these results confirms that αPEP25h specifically recognizes iPmel17.

Because Pmel17 is cleaved into two fragments by a proprotein convertase (5), we examined whether αPEP25h detected only iPmel17 and/or the cleaved N-terminal fragment (cNTF) of Pmel17. Thus, we treated MNT-1 cells with 50 μM Dec-RVKR-CMK (CMK), a known inhibitor of proprotein convertases, including furin (39), for 24 h and then metabolically pulse-chase-labeled them with \(^{35}\)S-Met/Cys. Pretreatment with CMK eliminated the cPmel17 form detected by αPEP13h (Fig. 1F) and actually stabilized mPmel17, as expected. In contrast, αPEP25h continued to detect only iPmel17. Digestion with EndoH shifted the mobility of iPmel17 as detected by αPEP13h and by αPEP25h.

Taken together, αPEP25h specifically recognizes an epitope of iPmel17 that is masked in mPmel17, which we hypothesize to result from steric hindrance of the epitope by glycosylation, as discussed below. αPEP25h works well in all immunological methods tested, an advantage over limitations in these proce-
FIGURE 1. αPEP25h detects Pmel17 in melanocytic cells. A, schematic showing the location of the αPEP25h epitope in the peptide sequence of Pmel17 and potential N-glycosylation sites at residues 81, 106, 111, 321, and 568. Potential O-glycosylation sites are present at both ends of the αPEP25h epitope at residues 393 and 414; the location of the αPEP13h epitope at the C terminus is also shown. NTD, N-terminal domain; PKD, polycystic kidney disease domain. B, MNT-1 cells were labeled for 30 min with [35S]Met/Cys and were chased for the times indicated (in min), after which samples were immunoprecipitated with αPEP13h or αPEP25h as noted. Immunoreactive bands were analyzed by SDS-PAGE and visualized by autoradiography as detailed under “Materials and Methods.” αPEP13h precipitated three bands: mPmel17 (white arrowhead), iPmel17 (black arrowhead), and cPmel17 (gray arrowhead), whereas αPEP25h identified only iPmel17 (black arrowhead). C, lysates were obtained from melanoma and from non-melanoma cells as noted and were immunoblotted with αPEP13h or αPEP25h as noted; β-actin was used as a loading control. D, lysates of MNT-1 cells were digested with EndoH or PNGaseF for 3 h at 37 °C, after which samples were immunoprecipitated with αPEP13h or αPEP25h as noted. Immunoprecipitated bands were visualized by autoradiography. E, cells metabolically labeled with [35S]Met/Cys were chased for 45 min and were sequentially immunoprecipitated with αPEP13h or αPEP25h, or with normal preimmune serum, as indicated. F, cells were treated with 50 μM CMK for 24 h and were then metabolically labeled with [35S]Met/Cys and chased for 1.5 h. Extracts were digested with EndoH where noted for 3 h at 37 °C, after which samples were immunoprecipitated with αPEP13h or αPEP25h as noted. Immunoprecipitated bands were visualized by autoradiography.
dures with the monoclonal antibodies to Pmel17 (HMB-50 and HMB-45).

**αPEP25h Detects iPmel17 in Early Melanosomes**—To evaluate the usefulness of αPEP25h in characterizing the intracellular localization and specificity of iPmel17, we performed immunofluorescence and immunoelectron microscopic analysis. Confocal microscopy (Fig. 2A) revealed that in MNT-1 cells, αPEP25h (top) had a granular cytoplasmic staining pattern and a linear distribution near the plasma membrane in 100 and 90% of cells observed, respectively. There was only a minor amount of colocalization of αPEP25h with HMB-45 (Fig. 2A, top left), a stage II melanosome marker, or with clathrin (top right), which was usually near the plasma membrane or in the perinuclear area but not in dendrites. These results indicate that αPEP25h detects iPmel17 after it leaves the ER compartment. Immunofluorescence of paraffin-embedded skin specimens unexposed to light (Fig. 2A, middle) or exposed to UV radiation (bottom) revealed that αPEP25h staining was restricted to melanocytes in the basal layer of the epidermis, where it showed a 97% colocalization with MART1, a melanocyte-specific marker, but not with keratin 5, a keratinocyte-specific marker. Interestingly, αPEP25h also revealed a fine extracellular granular pattern that was observed in or near suprabasal keratinocytes (Fig. 2A, insets, arrows), which may represent secreted vesicles (exosomes) or melanosomes containing iPmel17. Indeed, Pmel17 has been identified previously as a component of exosomes released by melanoma cells (40). Tissue sections from kidney, breast, lung, and uterus were processed in parallel with skin sections but showed no staining with αPEP25h (data not shown).

To further confirm the intracellular location of αPEP25h, we performed dual immunoelectron microscopy (Fig. 2B). αPEP25h (20 nm gold) was localized lining the internal matrix of stage I melanosome membranes and occasionally colocalized

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**TABLE 1**

| Peptides identified by mass spectrometry | Pmel17 peptides identified | Peptide position | Antibody | PEP25h | PEP13h |
|------------------------------------------|-----------------------------|-----------------|----------|--------|--------|
| NQDWLGVSRR                               | 29–37                       | 95              |
| QLYPEWTEAQR                               | 47–57                       | 95              |
| TWGQYWQVLGPGSVGLIGNGSR                   | 155–176                     | 99              |
| QVPLDCVLYR                                | 470–479                     | 95              |
| IFCSIPGENSPLLSEQQV                       | 643–661                     | 99              |

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**FIGURE 2. αPEP25h detects Pmel17 in stage I melanosomes.** A, MNT-1 cells were fixed and dual stained with αPEP25h (red) and with HMB-45 or clathrin (green). Note the granular distribution pattern of αPEP25h and the lack of colocalization (yellow) with HMB-45 (top row). Skin sections unexposed or exposed to UV light (middle and bottom, respectively) were deparaffinized and stained with αPEP25h (red) and for MART1 or keratin 5 (green). Note the colocalization of αPEP25h and MART1 (inset, left) and the presence of granular staining outside melanocytes (insets, arrows). B, immunoelectron microscopy of MNT-1 cells double-stained with αPEP25h (25 nm gold) and for AP2, HMB-50, or MART1 (10 nm gold). Colocalization of αPEP25h with AP2 and localization at the plasma membrane or exocytic vesicles are indicated with arrows. A vesicular exosome containing αPEP25h is shown in the inset. Roman numerals refer to melanosome stages.
with AP2-containing vesicles (10 nm gold). αPEP25h was also identified at the plasma membrane and within small vesicles (80 nm) near the plasma membrane that resemble exosomes (Fig. 2B, arrow) (16). In addition, αPEP25h colocalized with HMB-50 (10 nm gold) in stage I melanosomes and with MART1 (10 nm gold) in stage II melanosomes. The specific localization of αPEP25h reactivity in those organelles can be seen in several additional images (supplemental Fig. 3). Thus, we conclude that αPEP25h specifically recognizes and identifies iPmel17 localized on the internal side of stage I and II melanosomal membranes and in the plasma membrane, which confirms the sorting of this protein through the secretory pathway to melanosomes (14). Reactivity of αPEP25h with intramelanosomal fibrils may reflect the loss of the epitope by cleavage (19) or because of melanin deposition, or it may indicate that iPmel17 is not incorporated into those fibrils.

The iPmel17 Form Is Not Retained in the ER but Is Processed Differently in the Golgi—iPmel17 has been considered either as an ER-retained form, because of its sensitivity to EndoH digestion (5, 38), or as the cNTF-Pmel17 form, because of the predicted size of that fragment after cleavage (38). To resolve if iPmel17 represents either of those forms, we examined whether high mannose residues, an indication of ER location, were present on iPmel17 immunopurified using αPEP13h from MNT-1 cells. This purified iPmel17 sample was treated with or without a combination of three enzymes that remove hybrid and complex glycans as follows: neuraminidase (which removes sialic acid); β-1,4-galactosidase, which digests unmodified β-(1–4)-linked Gal; and β-N-acetylglucosaminidase, which removes β-linked GlcNAc (supplemental Fig. 4). Those samples were stained with the lectin Galanthus nivalis agglutinin, which recognizes high mannose type glycans. Carboxypeptidase Y, which contains only high mannose glycans, was used as a positive control. G. nivalis agglutinin reacted only with enzyme-digested iPmel17 (~80 kDa). These results confirm that neither iPmel17 nor mPmel17 contains high mannose glycans, showing that iPmel17 is not an ER-retained form and that it contains N-glycan structures normally modified in the Golgi. Furthermore, the difference in size between the band detected (~80 kDa) and its predicted size (~70 kDa) may be due to the presence of O-glycans added in the Golgi as discussed below.

To further assess those structures, we used enzymes that remove complex and hybrid structures, with or without neuraminidase treatment (Fig. 3A). As detected by αPEP25h, treatment with neuraminidase plus β1,4-galactosidase (Fig. 3A, lane 4) and those two enzymes plus β-N-acetylglucosaminidase
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FIGURE 4. Sialylation of O-glycans modulates the reactivity of αPEP25h. A and B, cell lysates from MNT-1 cells were digested in the presence or absence of the enzymes noted above each lane and were then analyzed by Western blot using αPEP25h, HMB-45, αPEP13h or peanut agglutinin. Samples were separated for 2 h on 10% Tris-glycine gels to maximize the separation of bands. Note the different bands appearing (arrows) after digestion with enzymes in lane 4. C, lysates of MNT-1 cells were immunopurified with αPEP13h or αPEP25h and were then digested with neuraminidase or PNGaseF as noted; bands reactive to D. stramonium agglutinin (DSA) were detected by Western blot. IP, immunoprecipitation.

(lane 5) generated novel bands (asterisks). In contrast, detection with αPEP13h revealed that treatment with β-N-acetylgalacosaminidase alone generated a strong band below iPmel17 at ~75 kDa (Fig. 3A, lane 3), which represents a net loss of ~25 kDa. These results suggest that αPEP25h recognizes a form of Pmel17 that is terminated at least galactose in a Gal-GlcNAc structure, whereas αPEP13h detected N-glycans terminated with GlcNAc (not extended with Gal or sialic acid). These data support the existence of similarly sized iPmel17 forms, not nated with GlcNAc (not extended with Gal or sialic acid). These

To evaluate whether iPmel17 corresponds to the cNTF form of Pmel17, we made two cDNA plasmids containing the full-length (FL) or the cNTF sequences of Pmel17, both being modified with a V5-His tag at their C-terminal domain (Fig. 3B). Those constructs were transiently transfected into MNT-1 cells. Immunoblotting analysis (Fig. 3C) revealed that cNTF-Pmel17 appeared as an ~75-kDa band (lanes 1 and 2), which was smaller than expected for the fully processed form (~85 kDa based on size of the predicted protein and its possible post-translational modifications). In contrast, FL-Pmel17 appeared as strong ~95-kDa bands and weak ~110-kDa bands (Fig. 3C, lane 4). The ~28-kDa band of cPmel17 was only observed after prolonged exposure (Fig. 3C, bottom, gray arrowhead, lanes 3 and 4). β-Actin was used as a loading control. Next, we deter-

larded, which is another type of post-translational modification that may alter the properties and functions of glycoproteins. It has been suggested recently that O-glycosyla-
tion occurs at the repeat domain (RPT) of Pmel17 (19). This creates glycoepitopes detectable by antibodies that have a double specificity toward both the peptide backbone and the carbohydrate structure. Therefore, we investigated the presence of O-glycans on iPmel17, as detected by αPEP25h. Samples were digested with neuraminidase or with endo-α-N-acetylgalactosaminidase (O-glycanase), which removes unmodified core 1 type O-glycan structures (Fig. 4A). αPEP25h detected only the single iPmel17 band after digestion with neuraminidase or O-glycanase, but multiple bands were detected after digestion with both enzymes (Fig. 4A, lane 4). Those multiple bands resembled that pattern detected by HMB-45 in undigested extracts (Fig. 4A, lane 1), although reactivity with HMB-45 was abrogated by neuraminidase digestion (lanes 2 and 4). This result indicates the presence of sialylated core 1 O-glycans on iPmel17. Interestingly, αPEP13h detected a band shift of mPmel17 after digestion with both enzymes (Fig. 4A, lane 4). To confirm that, Pmel17 immunopurified using αPEP13h was stained with peanut agglutinin that recognizes unmodified core 1 (Galβ1-3GalNAc-R) in O-glycans. Peanut agglutinin detected bands in samples digested with neuraminidase with or without O-glycanase (Fig. 4A, lanes 2 and 4). Note the disappearance of mPmel17 in Fig. 4A, lane 4. These results indicate that both mPmel17 and iPmel17 contain sialylated core 1 O-glycans and constitute further evidence that iPmel17 is processed through the Golgi but in a fashion distinct from mPmel17.
Because αPEP25h detects only iPmel17 and not mPmel17, we hypothesized that is because of O-glycan modification. To confirm that O-glycosylation, but not N-glycosylation, masks the αPEP25h epitope on Pmel17, we removed all N-glycan structures with PNGaseF followed by neuraminidase and O-glycanase digestion (Fig. 4B). Immunoblotting analysis revealed that iPmel17, as detected by αPEP25h, was further reduced to the size of the predicted peptide (75 kDa) after digestion with all three enzymes (Fig. 4B, arrow, lane 4). In contrast, staining with HMB-45 revealed a similar pattern for iPmel17 (Fig. 4B, upper band), indicating that this particular form still contains some sialylated O-glycans, although those bands were far less intense than those seen at the bottom of the gel. In contrast, all bands below iPmel17 were PNGaseF-resistant, showing their lack of N-glycan structures. Thus, we hypothesize that N-glycosylation in the ER affects only a small proportion of the Pmel17 and partially impairs its subsequent O-glycosylation in the Golgi, hence making the epitope available for detection by αPEP25h. To examine the presence of complex N-glycans, Pmel17 immunopurified with αPEP13h or with αPEP25h was digested with neuraminidase and/or PNGaseF and then was stained with the lectin Dathura stramonium agglutinin, which binds galactose in Galβ1–4GlcNAc-R structures in unsialylated complex type N-glycans and partially extended core 2 O-glycans (Fig. 4C). D. stramonium agglutinin detected bands in undigested Pmel17 immunopurified with αPEP13h or αPEP25h (Fig. 4C, lanes 1 and 5) and in neuraminidase-digested samples (lanes 2 and 6), but not in PNGaseF-treated samples. This indicates that iPmel17, and to a lesser extent mPmel17, contains some hybrid or complex type N-glycans that are not modified with sialic acid in MNT-1 cells. Asia-}

Sialylation Influences the Stability and Sorting of Pmel17—Our data indicate that sialic acid is added mostly to core 1 O-glycans of Pmel17 in MNT-1 cells. Therefore, we further investigated the role of sialic acid in the processing of Pmel17. First, we inhibited the addition of sialic acid by blocking the enzyme activity of ST3Gal I, the most common sialyltransferase active on core 1 O-glycans, with the specific inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) overnight and then metabolically labeled MNT-1 cells with [35S]Met/Cys for 30 min (Fig. 5A). As detected by αPEP25h and by αPEP13h, treatment with DANA reduced the band intensity and stability of all Pmel17 bands after a 90-min chase compared with the control (Fig. 5A, lane C). To determine whether Pmel17 degradation occurs in a pre- or post-Golgi compartment, we treated cells overnight with DANA and then metabolically labeled them with [35S]Met/Cys for 30 min (Fig. 5A). As detected by αPEP25h and by αPEP13h, treatment with DANA reduced the band intensity and stability of all Pmel17 bands after a 90-min chase compared with the control (Fig. 5A, lane C). To determine whether Pmel17 degradation occurs in a pre- or post-Golgi compartment, we treated cells overnight with DANA and then metabolically labeled them with [35S]Met/Cys for 30 min. Following that, we added brefeldin A (BFA, an agent that blocks anterograde traffic from the Golgi) or MG132 (a proteasome inhibitor) (Fig. 5B). The addition of either of those compounds increased Pmel17 stability after a 90-min chase, as detected by αPEP25h and by
αPEP13h, which suggests that degradation of Pmel17 occurs in a post-Golgi compartment.

Because there is evidence that sialylated O-glycans can carry plasma membrane apical sorting information (41, 42), we analyzed the intracellular distribution of Pmel17 in melanoma cells treated with DANA using immunofluorescence (Fig. 5C). Surprisingly, αPEP25h and αPEP13h (Fig. 5C, red) showed reduced signals and distributions restricted to the perinuclear area where it colocalized moderately with the Golgi marker Vti1b (green). In contrast, cells treated with DANA plus BFA or MG132 (Fig. 5D) showed a recovery of the iPmel17 signal detected by αPEP25h (red). Note the colocalization of Pmel17 with BIP (an ER marker, Fig. 5D, green) after treatment with BFA and its localization in dendrites after treatment with MG132. Taken together, these results indicate that the addition of sialic acid on core 1 type O-glycans is important for the stability of Pmel17 and for its correct sorting through the secretory pathway.

Pmel17 Glycoforms Deficient in Sialic Acid and Galactose Lose the Ability to Form Fibrils—Ever since it was first demonstrated that fibrils within stage II melanosomes react positively with HMB-45, which recognizes an epitope modified with sialic acid (4, 43), it has been assumed that sialic acid plays a role in fibril formation. However, αPEP25h detects band patterns similar to HMB-45 after treatment with neuraminidase, which suggests that sialic acid alone may not be critical for fibril formation. To address this, we transfected Pmel17 into CHO cells and into Lec2 and Lec8 mutants of CHO cells with disrupted glycan functions, and we evaluated the processing and fibril formation capability of Pmel17. Lec2 mutant CHO cells have a deletion mutation in the CMP-sialic acid transporter which results in 90% decrease in sialic acid content (44). Lec8 mutant CHO cells have a deletion mutation in the UDP-galactose transporter which results in a truncated protein with a greatly reduced ability to translocate UDP-Gal inside the Golgi (45). Thus, Lec8 cells generate nonsialylated and nonsialylated N- and O-glycans (46). Immunoblotting analysis of those transfected cells revealed differences in the glycan structures of Pmel17 in these cells was assessed using digestion with EndoH or PNGaseF (supplemental Fig. 5). mPmel17 and iPmel17 were insensitive or sen-
sensitive to EndoH in all three cell lines, respectively, as expected. In contrast, all Pmel17 bands were sensitive to PNGaseF, also as expected. These results indicate that the mPmel17 and iPmel17 glycoforms expressed in CHO cells are processed similarly as in human melanocytic cells.

To analyze modifications to N- and O-glycan chains, we took advantage of the fact that Lec2 cells are unable to add GalNAc to complex-type N-glycans (47) and that Lec8 cells only form O-GalNAc (48) (Fig. 6B). As detected by αPEP25h, digestion with neuraminidase and O-glycanase produced an ~80-kDa band (Fig. 6B, asterisk) in CHO cells (lane 3). A similar band was observed in Lec2 cells only after digestion with O-glycanase alone (Fig. 6B, lane 2), whereas no similar changes were observed in Lec8 cells. These results further confirm that iPmel17 is modified with sialic acid and that ~10 kDa of core 1 O-glycans are added. Analysis of Pmel17 with αPEP13h revealed that the same combination of neuraminidase and O-glycanase reduced the size of mPmel17 (Fig. 6B, “<”), as had been seen in MNT-1 cells (compare with Fig. 4A). Interestingly, αPEP13h detected a pair of novel bands after digestion with neuraminidase, β1,4-galactosidase, and β-N-acetylglucosaminidase (Fig. 6B, lane 10, arrows). Thus, these data indicate that two differently glycosylated forms of Pmel17 are produced, and those forms have similar sizes to iPmel17 as detected in MNT-1 cells. These results are consistent with our previous observations that iPmel17 is a glycoform of Pmel17.

The primary biological role of Pmel17 is the formation of fibrils within melanosomes, which is essential for the maturation and pigmentation of those organelles. Our group has reported that MART1 interacts with Pmel17 and facilitates fibril formation in transfected cell lines (32). Therefore, Pmel17 and MART1 were transiently transfected together into CHO, Lec2, and Lec8 cells and were analyzed by electron microscopy (Fig. 6C). In mock-transfected cells, large multivesicular bodies containing electron-dense material were frequently observed in all three cell lines. Transfected CHO and Lec2 cells showed fragments of organized fibrils (Fig. 6C, insets, arrowheads) within multivesicular bodies, but those structures were observed less frequently in Lec2 cells. However, the formation of thick fibrils was observed only in Lec2 cells (supplemental Fig. 6), suggesting that the lack of sialic acid promotes the accumulation of Pmel17 without its further organization. Surprisingly, we were unable to identify any fibrils or similar structures in Lec8 cells. The sum of these results suggests that the addition of both sialic acid and galactose to Pmel17 is critical to its capacity to form fibrils and that those changes may be related to the capacity to form sialylated core 1 O-glycans.

Transfer of Sialic Acid to Core 1 O-Glycans Is Reduced in MNT-1 Melanoma Cells—Our results showing that O-glycan chains on Pmel17 are usually modified with sialic acid, whereas N-glycan chains are not, suggest a more efficient transfer of sialic acid to these O-glycan structures. The addition of sialic acid to nascent
O-glycan chains stops the further extension of those structures. Changes in sialyltransferase expression and activity have already been associated with the regulation of glycoepitopes (49). Interestingly, high metastatic potential has been associated with increased β1,6-branching in N-glycans of melanoma cells (50, 51). Thus, we hypothesize that melanoma cells may exhibit altered sialyltransferase activities compared with normal melanocytes, which would affect the glycosylation pattern of most proteins, including Pmel17. To examine that possibility, we checked the expression of ST3Gal I and ST6GalNAc II, which commonly act on core 1 O-glycan structures (Galβ1–3GalNAcα-R). Quantitative reverse transcription-PCR showed that mRNAs encoding both sialyltransferases were expressed by melanocytic cells (supplemental Fig. 7). We then analyzed levels of sialyltransferase activity in MNT-1 cells compared with normal human melanocytes using different glycoproteins as acceptor substrates. As shown in Fig. 7A, both types of cells had sialyltransferase activity toward asialofetuin, which contains unsialylated N-glycans and unsialylated core 1 O-glycans, although MNT-1 cells were less active than normal melanocytes. In contrast, very high levels of sialyltransferase activity were detected using fully sialylated fetuin as an acceptor, showing that these cells can substitute sialic acid on already sialylated carbohydrates, an ability known as oligo- or polysialylation (52). Thus, normal melanocytes are 5 times more active than MNT-1 cells and 25 times more active than the breast cancer cell line T47-D, which expresses ST3Gal I and ST6GalNAc II (53), used as controls (data not shown). To discriminate between sialic acid transferred to N-glycans or to O-glycans, fetuin and asialofetuin were treated with PNGaseF, which allows the precipitation of proteins without N-glycans and the measurement of radioactivity transferred only to O-glycans (Fig. 7B). Note the reduced levels of sialic acid after treatment with PNGaseF, which corresponds to sialic acid transferred to N-glycans in asialofetuin and in fetuin. Nevertheless, normal melanocytes still exhibited four times more sialyltransferase activities toward fetuin than did MNT1 cells. This result also suggests that normal melanocytes express some polysialyltransferase (ST8sia) active on sialylated O-glycans that is down-regulated in the melanoma cells. We then assessed the transfer of sialic acid to N- or O-glycans using the following acceptors: asialo-orosomucoid, orosomucoid (which contains only N-glycans), BSM, and asialo-BSM (which carries core 3 O-glycans) (supplemental Fig. 8). Normal melanocytes and MNT1 cells exhibited low levels of activity toward N-glycans with or without sialylation, as assessed using orosomucoid and asialo-orosomucoid as acceptors, respectively. Therefore, we concluded that normal melanocytes and MNT-1 cells preferentially transfer sialic acid to core 1 O-glycan structures.

To further confirm these novel findings, we used immunoblotting to analyze the products of fetuin and asialofetuin with or without PNGaseF digestion in lysates of T47-D cells (as a positive control), NHM, MNT-1 cells, and water (as a negative control) (Fig. 7B). Ponceau staining showed various patterns that reflect differences between cell lysates, except in Fig. 7B, lanes 4, 8, 12, and 16, which contained only fetuin. Lectin staining with *Maackia amurensis* agglutinin, which recognizes α2,3-linked sialic acid to galactose, revealed key differences between normal melanocytes and MNT-1 cells. Surprisingly, α2,3-linked sialic acid was detected only in N-glycans of normal melanocytes (Fig. 7B, lanes 6 versus 10 and 14 after PNGaseF digestion). In contrast, α2,3-sialylation was present both in N-glycans and in O-glycans of MNT-1 cells (Fig. 7B, lanes 7 versus 11 and 15). Note the removal of α2,3-linked sialic acid after PNGaseF digestion (Fig. 7B, lane 12) from the N-glycans in fetuin (lane 4) indicates the specificity of the method. In addition, detection of 1H-labeled sialic acid on the membrane using a PhosphorImager confirmed that most sialic acid transferred during the assay was transferred to O-glycans because the intensity of the signal did not change after PNGaseF digestion (Fig. 7B, right versus left half). Note that asialofetuin can be an acceptor for sialyltransferases in these cell lines, but the transfer is very low compared with fetuin. The high levels of sialyltransferase activity, their predilection to transfer α2,3-sialic acid to N-glycans, and the reduced stability of Pmel17 after inhibition of α2,3-linked sialic acid transfer to core 1 O-glycans suggest that treatment with DANA would...
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Affect melanogenesis, especially in normal melanocytes. Thus, normal melanocytes were untreated or were treated with DANA for 6 days, and the melanin contents in the cell pellets were then measured (Fig. 7C). Interestingly, a highly significant 20% decrease ($p < 0.001$) in melanin content was observed following treatment of normal melanocytes with 20 μM DANA. This result probably reflects an overall reduction in melanosome formation because of the decreased stability of Pmel17.

However, we cannot exclude that melanin forming enzymes, e.g. tyrosinase, may also be affected.

Taken together, these results demonstrate that MNT-1 melanoma cells have reduced levels of sialyltransferase expression and activity compared with normal melanocytes. Most importantly, we determined that normal melanocytes and MNT1 cells preferentially transfer sialic acid to O-glycan chains.

**DISCUSSION**

The design and specificity of αPEP25h allow for a better understanding of the complex processing, sorting, and trafficking of Pmel17. In depth analysis of post-translational modifications in Pmel17 has been long overdue. αPEP25h is the first antibody that recognizes a known peptide sequence in the luminal domain of Pmel17, which constitutes an advantage compared with monoclonal antibodies such as HMB-45 or HMB-50 that recognize undefined epitopes (54, 55). Because the peptide sequence recognized by αPEP25h is at the RPT domain, we confirmed that extensive O-glycosylation, as we suppose occurs in mPmel17, hides the epitope and thus abrogates reactivity by αPEP25h (as proposed in Fig. 1A). This constitutes an advantage to study the dynamics of iPmel17, which was previously and incorrectly proposed to represent an ER form of Pmel17 based only on its sensitivity to EndoH. Interestingly, iPmel17 is not the only melanosomal protein with this type of sensitivity to EndoH digestion, because similar patterns have been described for correctly processed, although inactive, tyrosinase (56) and for DOPACHrome tautomerase (57). Fig. 8 presents a schematic of Pmel17 processing through cytoplasmic compartments.

The various results obtained with αPEP25h in this study confirm that iPmel17 is not retained in the ER but is glycosylated in a manner distinct from mPmel17. Interestingly, immunoelectron microscopy with αPEP25h detects iPmel17 on the inner side of early stage melanosomal membranes. These results indicate that iPmel17 is intact, not cleaved, at the time it arrives to stage I melanosomes, an observation consistent with the highly amyloidogenic properties of cNTF-Pmel17 (58).

In this study, we found evidence that N-glycans can be differently N-glycosylated and sialylated in Pmel17, and we have clearly demonstrated that all its glycoforms are substituted with sialylated core 1 O-glycans, although the exact structure of those O-glycans remains to be determined. Interestingly, high metastatic potential of melanoma cells has been associated with increased β1–6 branching of N-glycans, because of high activity of N-acetylglucosaminyltransferase V (50, 51). This constitutes an altered processing of N-glycans, and one could thus address the question about the impact of such an alteration on Pmel17 membrane expression in melanoma cells.

In light of our results, it is also critical to understand the role of high mannose/hybrid-type glycans in iPmel17 and melanoma progression. In murine melanoma cells, proteins modified with mannose-type glycans interact with cell surface lectins that are necessary to initiate the spread of murine melanoma (59, 60) and its metastasis to the liver (61). Furthermore, high mannose-type glycans are also involved in the functionality and cell surface expression of the mature human transferrin receptor (62, 63). Therefore, the mannose-rich/hybrid type N-glycans on iPmel17 may play an active role in its sorting to the cell surface and a similar mechanism involving a mannose-binding lectin may also be involved in its active internalization. The overall reduction in sialyltransferase activity and the predilection to sialylated O-glycan chains in melanoma cells provide for the first time a mechanistic basis for these processes in melanocytic cells.

These findings also reveal a new factor to consider in the processing of melanoma-specific proteins. Differences in the pattern of expression of sialyltransferases, such as ST3 Gal I, between normal and malignant cells had already been associated with a cancer-specific regulation of glycoepitopes (49). In addition, the activities of sialyltransferases, although high in these cells, may be regulated both in normal and in malignant cells by factors that also regulate pigmentation, such as α-melanocyte-stimulating hormone. Sialic acids are often present at the nonreducing ends of glycans, conferring strong negative charges on the protein. Melanins are polyanions with a relatively high content of negatively charged carboxyl groups and ortho-semiquinones at physiological pH (64). In this context, how does sialic acid addition then influence the structural role of Pmel17 and its ability to bind melanin to melanosome fibrils? We hypothesize that sialic acid, when added to N-glycans on Pmel17, makes it hydrophilic and leads to the exposure of hydrophobic protein domains, as occurs after the cleavage of Pmel17; this would then favor the formation of polymers through hydrophobic interactions. In contrast, sialic acid added to O-glycans may have a dual role as follows: 1) carrying plasma membrane sorting signals, and 2) protecting Pmel17 against early cleavage and degradation, a function that has been reported previously in other cell lines (41, 42). Several studies support these roles as follows. The extent and type of O-glycans modified with sialic acid in the RPT domain of Pmel17 play key roles to determine the proper glycoform of Pmel17 destined to form fibrils (19) or to be targeted to the plasma membrane (41, 42). Interestingly, the cNTF fragment reconstituted from E. coli has been shown to be amyloidogenic (58). In this context, it seems that glycosylation may not be required but in fact glycosylation and especially sialylation regulate this process actively (65). Our findings in this study indicate that in addition to sialic acid, galactose also plays a role in regulating fibril formation, but it may contribute in various ways. Pmel17 sorted to the plasma membrane may have a counter-receptor that interacts both with the protein and with its glycan moieties similar to what has been shown for the interaction of P-selectin with its ligand (66) or the mannose 6-phosphate receptor system that targets proteases to lysosomes (67). On the other hand, the presence of sialic acid and galactose could stabilize Pmel17 to

4 J. Valencia, unpublished results.
achieve a defined conformation that may favor its safe transport
and avoid polymerization. Such a stabilizing effect by sialic acid
on protein structure has been observed previously (68).

A fair question to ask is why do melanocytes require different
types of Pmel17? We hypothesize that this reflects the multi-
functional nature of Pmel17. Once mPmel17 is processed and
trafficked to stage I melanosomes, it is immediately processed
and its C-terminal and cNTF fragments are cleaved to allow
fibril formation (19). Following that, melanin is deposited on
the fibers, and it is essentially trapped and covered by melanin.
Despite that, Pmel17 is also present on the plasma membrane
and is one of the most common melanoma antigens detected.
Thus, Pmel17 secreted or at the plasma membrane may play
other roles, which will require further study to determine. With
that in mind, glycosylation seems to play a critical role in deter-
mieving the trafficking of Pmel17 to various subcellular com-
partments and thus regulates its functions. Some glycosylated
forms of Pmel17 (i.e. mPmel17) will sort directly to melan-
osomes to initiate melanosome biogenesis, whereas other glyco-
sylated forms (i.e. iPmel17) are sorted through the secretory
pathway for secretion or recycling.

Taken together, αPEP25h has proven to be a useful antibody
that allows specific detection of iPmel17 and allows character-
ization of its processing and trafficking. Our findings demon-
strate that iPmel17 is a glycosylated form that is distinct from
mPmel17 and that it is not an ER-retained form. Such differ-
ences and the distinct sorting patterns that result in the differ-
ent glycoforms raise the possibility that Pmel17 may have other
important functions in addition to its well known role in gen-
erating the structural fibrillar matrix of melanosomes. Our
novel findings confirm that the addition of sialic acid to O-
glycans on Pmel17 on melanosomes is involved in the stability and sorting of this
protein through the secretory pathway, and that the modific-
ration with sialic acid and galactose to Pmel17 glycans is critical to
its ability to form fibrils, a process that directly regulates pig-
mentation in mammals. Furthermore, alterations in sialyltrans-
ferase activity and substrates in melanoma cells provide a
mechanistic explanation for the microheterogeneity observed
in the glycan structures of Pmel17.

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