MART-1 Is Required for the Function of the Melanosomal Matrix Protein PMEL17/GP100 and the Maturation of Melanosomes*§

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More than 125 genes that regulate pigmentation have been identified to date. Of those, MART-1 has been widely studied as a melanoma-specific antigen and as a melanosomal-specific marker. Whereas the functions of other melanosomal proteins, such as tyrosinase, tyrosinase-related protein-1, dopachrome tautomerase, and Pmel17, are known, the function of MART-1 in melanogenesis, is unclear. A role for MART-1 in pigmentation is expected because its expression pattern and subcellular distribution is quite similar to the other melanosomal proteins and usually correlates with melanin content. We investigated the function of MART-1 using a multidisciplinary approach, including the use of siRNA to inhibit MART-1 function and the use of transfection to re-express MART-1 in MART-1-negative cells. We show that MART-1 forms a complex with Pmel17 and affects its expression, stability, trafficking, and the processing which is required for melanosome structure and maturation. We conclude that MART-1 is indispensable for Pmel17 function and thus plays an important role in regulating mammalian pigmentation.

Melanosomes are lysosome-related organelles which have the unique capacity to produce melanin pigment (1) and which progress through four sequential morphological steps as they mature (2). Stage I melanosomes are round, membrane-bound and electron-lucent vesicles that are generally found in the perinuclear area. The transition to Stage II melanosomes involves an elongation of the vesicle, and the appearance within distinct fibrillar structures. The production of those internal matrix fibers and the maturation from Stages I to II melanosomes depend on the presence of a structural protein termed Pmel17, also known as gp100 or SILV. Shortly after its delivery to Stage I melanosomes, Pmel17 is cleaved into several fragments, which form the fibrillar matrix of the organelle (3–6). In pigmented cells, melanins are deposited on these 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 little or no internal structure is visible, at which time they are termed Stage IV melanosomes. More than 125 pigmentation-related genes have been identified to date (7). Tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1), dopachrome tautomerase (DCT), Pmel17, and MART-1 (also known as Melan-A) are well known as melanosomal-specific proteins, and, with the exception of MART-1, all have specific and defined roles in melanogenesis.

MART-1 was initially cloned by two independent groups using melanoma reactive CD8+ T cells (8, 9). MART-1 is a type III membrane protein (with an apparent molecular mass of 22–24 kDa) localized in the endoplasmic reticulum (ER), in the trans-Golgi network (TGN) and in melanosomes (10–12). MART-1 does not contain consensus signals for N-linked glycosylation sites or for N-terminal signal peptides, which TYR, TYRP1, DCT, and Pmel17 possess. Although MART-1 has no apparent homology to other known melanosomal proteins and no detectable enzymatic activity, MART-1-1 is highly enriched in early melanosomes (Stage I and/or II melanosomes) (5, 10), which suggests that it might play some role in early melanogenesis and may be trafficked to melanosomes with other melanosomal proteins.

Pmel17 is the human homologue of murine silver, whose disruption of function produces a silver hair color in mice (13). Pmel17 was also cloned initially as a melanoma specific antigen recognized by tumor infiltrating lymphocytes (14). Both MART-1 and Pmel17 serve as common targets for tumor-directed T lymphocytes; both are often highly expressed in melanoma cells and have been widely studied as targets of immunotherapy for melanoma (15). However, despite intensive efforts, the role of MART-1 in melanocytes, and perhaps in melanogenesis, has remained unknown, and the phenotype caused by the loss of MART-1 function is undefined.

In this study, we assessed the role of MART-1 in human melanocytic cells, including normal human melanocytes and human melanoma cells. We used siRNA technology as well as transfection of MART-1-1 into MART-1-negative melanoma cells to characterize the function of MART-1. We show that MART-1 plays a vital role in the expression, stability, trafficking, and processing of Pmel17, which is critical to the formation of Stage II melanosomes. This study also provides important clues toward understanding the synergistic effects of melanosome-associated proteins and the role of MART-1 in pigmentation.

MATERIALS AND METHODS

Cell Cultures—MNT-1 cells and SK-MEL-28 cells were used as described previously (16). WM266-4 cells and HeLa cells were purchased from the ATCC (Manassas, VA). Primary normal human melanocytes, 1

1 The abbreviations used are: TYR, tyrosinase; AP, adaptor protein complex; BFA, brefeldin A; DCT, dopachrome tautomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MG132, benzoylcyano- bonyl-Leu-Leu-leucinal; NRS, normal rabbit serum; TYRP1, tyrosinase-related protein-1; ER, endoplasmic reticulum; siRNA, small interfering RNA; DAPI, 4′,6-diamidino-2-phenylindole.
a kind gift from Dr. Zalfa A. Abdel-Malek (University of Cincinnati) were cultured in melanocyte basal medium with growth supplements (Cascade Biologics, Portland, OR). At least three independent strains of melanocytes were used in each experiment reported in this study.

Plasmid Construction and Transfection—We reverse-transcribed total RNA from MNT-1 cells using a cDNA synthesis kit (New England Biolabs, Beverly, MA). cDNA was amplified with PCR cloning enzyme (Stratagene, La Jolla, CA) using MART-1-specific primers. Products were ligated into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Pmel17 vector was constructed by ligating an EcoRI/XbaI DNA fragment from hgp100 (hgp100 was kindly provided by Dr. Nicholas Restifo at the NCI, National Institutes of Health) into pcDNA3.1 vector (17). The constructs were confirmed by sequence analysis. As a negative control vector, the pcDNA3.1 vector with no insertion was used. For transfection, cells were plated 1 day in advance and transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

siRNA Studies—MART-1 siRNA was synthesized using the Silencer siRNA Construction Kit (Ambion, Austin, TX). The following DNA template and its complementary antisense sequence were used to make double-stranded MART-1 siRNA: aagcagagttgacagcag and its complementary sequence. For a negative control, we used aagcagaggaacaagctcg and its complementary tetragoplementary sequence. They were reverse-transcribed to make siRNAs. The following DNA strand and its complementary antisense sequence were used to make siRNA transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer’s instructions (2.0 μg/ml siRNA was used unless noted). We confirmed that these siRNAs did not share at least 21-bp successive sequences with any human Pmel17 mRNA sequence, which had ever been published.

Semiquantitative RT-PCR Analysis—We reverse-transcribed total RNA using Superscript III (Invitrogen). PCR reactions consisted of 35 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (15) and Pmel17, and 40 cycles for MART-1 using Ex-TaqHS (Takara). PCR products for GAPDH, MART-1 and Pmel17 were 450, 300, and 700 bp, respectively, and were electrophoresed in parallel with DNA molecular mass marks (Invitrogen). Each PCR product was also electrophoresed with a reaction after five additional cycles and was confirmed to be without contamination. The PCR products and their transcripts relative to GAPDH were quantified by Scion Image software (Scion, Frederick, MD). Each experiment was repeated three times.

Primers sequences are available upon request.

Antibodies and Reagents—αPEP1h, αPEP7h, αPEP8h, and αPEP13h antibodies were generated in rabbits against synthetic peptides corresponding to the C terminus of human TYRP1 (18), TYRP1, DCT, and Pmel17, respectively (6). M2-9E3 and M2-7C10 were used for MART-1 (Neo Markers, Fremont, CA), A103 for MART-1 (Santa Cruz Biotechnology), HMB45 for the processed internal domain of Pmel17 (DAKO, Carpinteria, CA), HMB50 for full-length Pmel17 (Neo Markers), anti-KDEL (Stressgen, San Diego, CA), anti-LAMP-2 (Research Diagnostics, Flanders, NJ), anti-Bip/GRP78, anti-Vti1b (BD Transduction Laboratories, Lexington, KY), HMB50 for full-length Pmel17 (Neo Markers), anti-KDEL (Stressgen, San Diego, CA), anti-LAMP-2 (Research Diagnostics, Flanders, NJ), anti-Bip/GRP78, anti-Vti1b (BD Transduction Laboratories, Lexington, KY), and anti-β-actin (Abcam, Cambridge, MA) were used. For immunopurification, a mixture of M2-9E3, M2-7C10 and A103 was used. M2-9E3 was used for other applications. BFA was purchased from Sigma. MG132 was purchased from Calbiochem.

Subcellular Fractionation—For purification of melanosomes, ER, and Golgi apparatus, we used the protocols previously described (16).

Immunofluorescence Microscopy—Cells were plated in 2-well Lab-Tek chamber slides (Nalge Nunc, Rochester, NY) and incubated at 37 °C and were stained by double or triple indirect immunofluorescence methods. After three washes in phosphate-buffered saline, the cells were fixed in 4% paraformaldehyde for 30 min at 4 °C. After three further washes in phosphate-buffered saline, the cells were permeabilized with 100% methanol for 15 min at 4 °C and were then blocked with 5% bovine serum albumin (Amersham Biosciences) for 1 h at room temperature. The cells were incubated with a mixture containing a polyclonal and/or monoclonal antibodies (at the dilution noted in the figure legends) overnight at 4 °C. After three washes in phosphate-buffered saline, the polyclonal antibodies were reacted with goat anti-rabbit IgG1 (1:400) labeled with Alexa Fluor 594 (Molecular Probes, Eugene, OR), and the monoclonal antibodies were reacted with goat anti-mouse IgG1 (1:200) and anti-mouse IgG1 (1:200) and anti-mouse IgG1 (1:200) labeled with Alexa Fluor 488, 594, or 647 (Molecular Probes). Then, the cells were counterstained with DAPI (Vector, Burlingame, CA). The reactivities of Alexa Fluor 488 and 594 were visualized as green and red signal, respectively. They were classified into three categories, according to whether they showed green, red, or yellow signals. The latter was indicative of colocalization of the red and green signals. The reactivities of Alexa Fluor 647 and DAPI are visualized as violet and blue, respectively. All preparations were examined with a confocal microscope (LSM 510; Zeiss, Germany), equipped with HeNe (543 nm and 633 nm), argon, and krypton laser sources.

Electron Microscopy—Electron microscopy was performed as previously described (16). Thin sections were examined using a Zeiss EM 912 Omega electron microscope.

Protein Extraction and Immunoblotting—Cell extracts were prepared, and immunoblotting was performed as described previously (6).

Metabolic Labeling and Immunoprecipitation—Radioactive metabolic labeling and immunoprecipitation were performed as described previously (6). Briefly, cells were starved in the Met/Cys-free medium for 30 min and then were pulse for 30 min with [35S]Met/Cys (Amer- sham Biosciences). They were chased for specific periods (as detailed in the figure legends) in medium containing 1 mM cold methionine, and then were solubilized in immunoprecipitation lysis buffer (6). If indicated, MG132 and BFA were added from the “starvation” and “pulse” periods, respectively, to the “chase” period. Cell extracts were incubated with normal rabbit serum (NRS; Vector) and were then incubated with protein G beads (Amersham Biosciences). The supernatants were incubated with αPEP13h. The immune-complexes were separated by incubation with beads and were further washed with immunoprecipitation lysis buffer. Thefinal pellets were eluted, electrophoresed, and visualized by autoradiography. For cold immunoprecipitation, samples were pulse-labeled with Met/Cys buffer and were then solubilized with appropriate controls or antibodies as detailed in the figure legends. The immunocomplexes were separated by incubation with beads and were further washed. Immunoblotting was then performed as described above.

Sequential immunoprecipitation was described previously with some modifications (21). The duration of the “starvation” and the “pulse” was 0 and 20 min, respectively. Cells were solubilized in immunoprecipitation lysis buffer. Samples were incubated with normal mouse IgG (Vector), and then were further incubated with beads. The supernatants were incubated with MART-1 antibodies or with normal mouse IgG and then were further incubated with beads. After washing, immune complexes were eluted with elution buffer (22). Samples were incubated with NRS then were further incubated with beads to eliminate nonspecific binding and excess antibodies. Supernatants were used for the second immunoprecipitation with the same procedure as described above.

Statistical Analysis—Data are presented as means ± S.D. Student’s t test was used. Values of p < 0.05 were considered significant.

RESULTS

Expression of Melanosomal Proteins in Various Types of Melanocytic Cells—To elucidate the function of MART-1, we screened a number of melanocytic cell lines to find one that does not express MART-1 but does express other melanosomal proteins. Unpigmented WM266-4 human melanoma cells have been reported to have undetectable levels of MART-1 (10). As MART-1-positive controls, we used pigmented MNT-1 melanoma cells, unpigmented SK-MEL-28 melanoma cells and normal human melanocytes. We then characterized the expression of various other melanosomal proteins, including TYR, TYRP1, DCT, and Pmel17 (as recognized by the αPEP13h antibody, which is specific for the carboxyl tail of the protein) (Fig. 1A). SK-MEL-28 cells and WM266-4 cells, both of which are unpigmented, were negative for TYRP1. WM266-4 cells expressed negligible levels of MART-1, as previously reported (10); however, the other three types of cells strongly expressed MART-1, as also reported previously (16).

The processed and cleaved forms of Pmel17, termed P100 (−100 kDa) and P26 (−26 kDa) were detected by αPEP13h, which recognizes the C terminus of Pmel17 in all four types of cells, although with different relative band intensities (Fig. 1A). The P100 form has been termed P1 or the full-length form while the P26 form has also been termed as Mβ (3, 6); the epitopes of the different antibodies to Pmel17 are depicted in Fig. 1B. A ~75-kDa band (P75) was clearly detected by αPEP13h in melanocytes and in MNT-1 cells, and that band represents the nascent form of Pmel17 (6). However, WM266-4 cells expressed the P100 and P26 forms of Pmel17 much
weaker than did the other types of cells. It should be noted that H9251 PEP13h reacts well with Pmel17 in Stage I melanosomes or in the ER but very poorly with Stage II melanosomes (5). In contrast, HMB45 reacts specifically with the cleaved internal domain of Pmel17 (which is found only in Stages II-IV melanosomes), but does not react with the intact Pmel17 found in Stage I melanosomes (5, 6); HMB45 thus serves as a specific marker for fibrillar melanosomes (Stages II-IV). The reactivity of HMB45 consists mainly of a 35-kDa band and several larger diffuse bands (6). Surprisingly, WM266-4 cells did not express the processed form of Pmel17 that is recognized by HMB45, although the other cells were strongly positive for that epitope (Fig. 1A). We confirmed that WM266-4 cells express MART-1 and Pmel17 mRNAs and that there are no mutations in those genes, at least in their coding regions, compared with published cDNA sequences (data not shown).

We previously established a subcellular fractionation technique using a stepwise sucrose density gradient to purify melanosomes at various stages from MNT-1 cells (5) and from SK-MEL-28 cells (16). In pigmented MNT-1 cells, the 0.8–1.2 M and the 1.6–1.8 M sucrose fractions contain Stage I-II and Stage III-IV melanosomes, respectively (5, 23). In contrast, in unpigmented SK-MEL-28 cells, the 0.8 M and the 1.4–1.8 M sucrose fractions contain Stage I and II melanosomes, respectively (16). As expected from the results above, neither MART-1 nor Pmel17 (recognized by HMB45; data not shown) was detected in any of those fractions from WM266-4 cells, including the ER- and the Golgi-enriched fractions (Fig. 1C). The enrichment of the ER and Golgi fractions was demonstrated by reactivities of those fractions to the organelle markers Bip/GRP78 and Vti1b, respectively. To validate the identity of those melanosome fractions, we detected TYR by immunoblotting, which is readily detectable in early melanosomes (16, 23). In WM266-4 cells, TYR was strongly detected in the 0.8–1.8 M sucrose fractions and in the ER (Fig. 1C). This reactivity pattern indicates that TYR trafficking is not significantly disrupted in WM266-4 cells. The P100 and P26 forms of Pmel17 were detected in the ER and in the 1.0–1.8 M sucrose fractions of WM266-4 cells, and the distribution was very similar to that of TYR. Diffuse, smeared bands detected by H9251 PEP13h were also observed in the 0.8–1.0 M sucrose fractions, which was likely due to degradation of Pmel17. There were no Stage II melanosomes (or even fibrils within vesicles) visible in the 1.0 or 1.8 M sucrose fractions by electron microscopy (Fig. 1D). Thus,
Pmel17 processing to the HMB45 reactive form is disrupted in WM266-4 cells since staining with HMB45 was always negative (Fig. 1A and other data not shown). Taken together, the patterns of expression of MART-1 and Pmel17 (recognized by HMB45) differed between WM266-4 cells and the other melanocytic cells. WM266-4 cells did not express detectable levels of MART-1 or Pmel17 (recognized by HMB45) in any sucrose fraction or in the cell lysate, although TYR and Pmel17 (recognized by P9251PEP13h) were readily detected. Those results indicate that the 1.0–1.8 M sucrose fractions in WM266-4 cells contain Stage I melanosomes.

Processing and Stability of Pmel17 in WM266-4 Cells—To characterize the processing of Pmel17 and to determine its stability in WM266-4 cells, we used pulse-chase metabolic labeling with P9251PEP13h (Fig. 1E). As a control, we used SK-MEL-28 cells, because they express other melanosomal proteins (TYR, DCT, and Pmel17) similar to WM266-4 cells and contain Stage II melanosomes (16). Pmel17 has been shown to be synthesized and glycosylated rapidly, then cleaved to the P26 form in MNT-1 cells (4, 6) and we confirmed that in SK-MEL-28 cells. The P120 form, which represents fully glycosylated Pmel17 and has also been reported as P2 (3), was detected between at 15 min to 1 h in both types of cells. Interestingly, a band noted as P75 (75 kDa) appeared after 30 min in SK-MEL-28 cells and it was even more apparent in WM266-4 cells, then chased for 0 min or for 45 min as noted. Extracts were immunoprecipitated with mouse IgG, MART-1 antibody, or P9251PEP13h; some were further immunoprecipitated with NRS or P9251PEP13h. Immunocomplexes were electrophoresed and visualized by autoradiography.

MART-1 Regulates Pmel17 Processing

Fig. 2. Detection of the MART-1/Pmel17 complex. A, lysates of melanocytes, SK-MEL-28 cells, and WM266-4 cells were immunoprecipitated with NRS, αPEP13h, αPEP7h (TYR), αPEP1h (TYRP1), or αPEP8h (DCT), as indicated. Immunocomplexes were analyzed by immunoblotting with the MART-1 antibody (M2-9E3). Alternatively (far right), samples were immunoprecipitated with normal mouse IgG or MART-1 antibody, and immune complexes were analyzed by immunoblotting with HMB45. Arrows indicate specific bands (nonspecific IgG bands that eluted from the column can also be seen). B, HeLa cells were transfected with mock, MART-1, Pmel17, or MART-1 + Pmel17 vectors. Expression of proteins in transfected cells were confirmed by immunoblotting (left panels) with antibodies to MART-1 (M2-9E3), β-actin, and Pmel17(αPEP13h), then were immunopurified as described above (right panel). C, subcellular fractions purified from SK-MEL-28 cells were analyzed by immunoprecipitation as described above. Immunoblotting with Bip/GRP78 (ER) and Vti1b (Golgi) are also shown. D, SK-MEL-28 cells were metabolically radiolabeled for 30 min, then chased for 0 min or for 45 min as noted. Extracts were immunoprecipitated with mouse IgG, MART-1 antibody, or αPEP13h; some were further immunoprecipitated with NRS or αPEP13h. Immunocomplexes were electrophoresed and visualized by autoradiography.
purified with αPEP13h but not in complexes immunopurified with antibodies to TYR, TYRP1, or DCT. As another control, MART-1 was not detected in similar extracts prepared from MART-1-negative WM266-4 cells. Conversely, we immunopurified proteins from melanocytes and from SK-MEL-28 cells with antibodies to MART-1 and then performed immunoblotting with HMB45. Specific bands showing the complex of MART-1 and Pmel17 were again clearly detected (Fig. 2 A), suggesting with HMB45. Specific bands showing the complex of MART-1 and Pmel17 were again clearly detected (Fig. 2 A), suggesting that complex.

We also examined the subcellular localization wherein the Pmel17-MART-1 complex is formed. To examine this, we prepared subcellular fractions from SK-MEL-28 cells and then immunoprecipitated Pmel17 with αPEP13h and then immunoprecipitated the absorbed proteins with an antibody to MART-1. In the ER, in the Golgi, and in the 0.8 and 1.0 M sucrose fractions (which contain Stage I and Stage I-II melanosomes, respectively), Pmel17-MART-1 immunocomplexes were clearly detected (Fig. 2C), but no such complexes were seen in fractions containing Stage II melanosomes only (1.4–1.8 M sucrose).

Pmel17 exists in several processed and cleaved forms (as shown above) and an interesting question is which form(s) of Pmel17 does MART-1 interact with? To resolve this, we performed sequential immunoprecipitation with metabolically labeled proteins. As shown above (Fig. 1E), only P100 is detected at 0 min, while in contrast, the P120, P100, and P26 forms are detected at 45 min. SK-MEL-28 cells were metabolically pulse-labeled for 30 min, and then were chased for 0 min or 45 min. Extracts of the labeled cells were then immunopurified with the MART-1 antibodies, then were further immunopurified with αPEP13h. Only the P100 form could be detected at 0 min and at 45 min (Fig. 2D). Neither the P120 form nor the P26 form was detected, even with prolonged exposure of the blot (data not shown). This result is consistent with the detection of the Pmel17-MART-1 complex in transfected HeLa cells as shown above, and its presence in the ER and Golgi fractions of SK-MEL-28 cells. Taken together, the results show that MART-1 and Pmel17 form a complex in an early subcellular compartment (in the ER and/or in the early Golgi). Moreover, MART-1 reacts with the P100 form and the HMB45 reactive form of Pmel17.

We next used confocal immunohistochemistry to examine the subcellular localization of MART-1 and Pmel17. MART-1 and Pmel17 (recognized by αPEP13h) colocalized in the perinuclear area in MART-1-positive cells (melanocytes and SK-MEL-28 cells), and again, WM266-4 cells were negative for MART-1 as expected (Fig. 3, top row). Moreover, in MART-1-positive cells, MART-1 and Pmel17 (recognized by HMB45) also colocalized in the perinuclear area (Fig. 3, middle row). We also confirmed the subcellular colocalization of MART-1 with full length Pmel17 (recognized by HMB50) (Fig. 3, bottom row). MART-1 has been previously reported to colocalize with Pmel17 recognized by NKI/beteb (12), an antibody, which also reacts with full length Pmel17 (6). Thus, we confirm in this study that MART-1 colocalizes with processed Pmel17 (recognized by αPEP13h and by HMB45) and show for the first time that MART-1 forms complexes with Pmel17.

**MART-1 Knockdown Reduces Pmel17 Expression and Abrogates the Processing of Pmel17**—We next designed MART-1-specific siRNA constructs to clarify the function of MART-1 with respect to the expression, stability, trafficking, and processing of Pmel17. MART-1 siRNA significantly reduced MART-1 expression in melanocytes and in SK-MEL-28 cells (Fig. 4A). Levels of the P100 and P26 forms of Pmel17 and reactivity with HMB45 were dramatically reduced by inhibiting MART-1 function with siRNA.
To clarify the level at which MART-1 affects Pmel17, e.g. within the ER or in a post-Golgi compartment, we used treatment with brefeldin A (BFA), which causes Golgi compartments to fuse with the ER and inhibits anterograde transport beyond the Golgi (24). We transfected MART-1 siRNA into MART-1-positive cells, then treated the transfected cells with BFA and immunoblotted with antibodies (M2–9E3, αPEP13h, HMB45, and β-actin). Arrows indicate specific bands. B, 48 h post-transfection, cells were further incubated for 6 h with or without BFA (at 1 μg/ml or 10 μg/ml for melanocytes or SK-MEL-28 cells, respectively), then analyzed by immunoblotting with αPEP13h. C, SK-MEL-28 cells were transfected with various concentrations of MART-1 siRNA as noted. Volumes used for siRNA treatments were adjusted to be identical with mock siRNA. Cells were analyzed as described in A. D, 48 h post-transfection, cells were metabolically radiolabeled and chased as described for Fig. 1E. Left, mock; right, MART-1. E, total RNAs were extracted from melanocytes or SK-MEL-28 cells harvested 48 h after transfection. Total RNAs were reverse-transcribed and amplified with GAPDH, MART-1, and Pmel17-specific primers. PCR products were electrophoresed and visualized with ethidium bromide (upper). Target gene transcripts relative to GAPDH were quantified (lower). Data graphed are means ± S.D. obtained from three independent experiments.

To clarify the level at which MART-1 affects Pmel17, e.g. within the ER or in a post-Golgi compartment, we used treatment with brefeldin A (BFA), which causes Golgi compartments to fuse with the ER and inhibits anterograde transport beyond the Golgi (24). We transfected MART-1 siRNA into MART-1-positive cells, then treated the transfected cells with BFA and immunoblotted with αPEP13h to examine the effect of MART-1 on Pmel17 within the ER (Fig. 4B). In mock-transfected cells, the P120 form of Pmel17 became a major band and P26 was significantly decreased by BFA. This result is consistent with the report that the cleavage of Pmel17 to the P26 form occurs in a post-Golgi compartment (4). P120 might represent a fully glycosylated form of Pmel17 associated with its retention in the ER and it was previously noted as P1* (3). These results also demonstrate that treatment with BFA abrogates Pmel17 trafficking and traps Pmel17 in the ER as well as abrogating Pmel17 processing in a post-Golgi compartment. P120 was clearly attenuated in both types of cells and P75 in melanocytes was also attenuated by MART-1 siRNA. These results support the hypothesis that MART-1 affects Pmel17 expression at the post-transcriptional level within the ER, and agree well with immunoblot experiments in the absence of BFA (Fig. 4A).

We further considered whether or not the reduction of HMB45 reactivity is simply due to the down-regulation of P100 and P26 forms. To resolve this, we transfected SK-MEL-28 cells with MART-1 siRNA at various concentrations. Down-regulation of P100 and P26 was observed only at higher concentrations of MART-1 siRNA (Fig. 4C). However, reduction of HMB45 reactivity was clearly observed at the lowest concentrations of MART-1 siRNA and reactivity with HMB45 was not reduced further at the higher concentrations. From these results, we conclude that the down-regulation of P100 and P26, and the reduction of HMB45 reactivity are independent phenomena. This result also shows that attenuation of Pmel17 (recognized by HMB45) is sensitive to even faint down-regulation of MART-1 function. We used pulse chase and immunoprecipitation analysis with αPEP13h to examine the stability of Pmel17 in mock or in MART-1 siRNA-transfected SK-MEL-28 cells. The stability of Pmel17 (P100) was significantly shortened by MART-1 siRNA (Fig. 4D).

We next examined MART-1 and Pmel17 mRNA levels following transfection with MART-1 siRNA. Semiquantitative RT-PCR analysis confirmed the reduction of MART-1 mRNA following transfection with MART-1 siRNA as expected, and showed further that Pmel17 mRNA levels were not significantly affected (Fig. 4E).

Reexpression of MART-1 Rescues Pmel17 Processing—To further characterize the involvement of MART-1 with Pmel17 expression and processing, we transfected a MART-1 expression vector into MART-1-negative WM266-4 cells and observed
the effects on Pmel17 and melanosome structure. MART-1 expression in those cells could now be clearly observed by immunoblotting (Fig. 5A). Interestingly, reactivity with HMB45 became detectable following the re-expression of MART-1, although the P100 and P26 forms of Pmel17 were only slightly enhanced. We also transfected mock or MART-1 expression vectors into WM266-4 cells, treated them with BFA and immunoblotted extracts with αPEP13h, as described above. P120 was clearly enhanced in MART-1 transfected WM266-4 cells (Fig. 5B). The P70 form, which was clearly detected in WM266-4 cells, disappeared in the presence of BFA. We again used pulse-chase metabolic labeling experiments with αPEP13h to examine mock or MART-1-transfected WM266-4 cells. The stability of Pmel17 (P100) was prolonged following the re-expression of MART-1 (Fig. 5C). These results are consistent with the results of the siRNA experiments described above.

At the cellular level, re-expression of MART-1 in WM266-4 cells resulted in the colocalization of MART-1 with Pmel17 (recognized by αPEP13h) in the perinuclear area of those cells (Fig. 5D), and Pmel17 (recognized by HMB45) now became detectable and clearly colocalized with MART-1. These expression patterns of Pmel17 are consistent with the inhibition of MART-1 function by siRNA seen in MART-1-positive cells, as described above. We conclude that Pmel17 expression, stability, and processing are affected by MART-1 at the post-transcriptional level. We also examined WM266-4 cells transfected with MART-1 at the ultrastructural level. WM266-4 cells contained few recognizable melanosomes with internal fibrils (Fig. 6A), but when MART-1 was re-expressed, those cells contained numerous organelles with striated matrices (Fig. 6B).

**Pmel17 Is Not Trafficked to Early Melanosomes Efficiently in the Absence of MART-1**—Pmel17 is delivered to Stage I melanosomes, although the specific trafficking route is still under discussion (4, 5). MART-1 is a membrane-bound protein, primarily localized in the Golgi, in the ER and in melanosomes (12). We hypothesized from our results above that MART-1 might assist Pmel17 in trafficking to early melanosomes. To examine this, we analyzed the subcellular localization of Pmel17 with organelle markers to clarify the effects of MART-1. In all three types of melanocytic cells, Pmel17 (recognized by αPEP13h) clearly colocalized with the ER marker KDEL (Fig. 7A). LAMP-2 serves as a lysosomal marker (25) and is also found in melanosomes (26, 27). LAMP-2 colocalized with Pmel17 (recognized by αPEP13h) in MART-1-positive cells (melanocytes and SK-MEL-28 cells) representing early melanosomes where Pmel17 is successfully trafficked in those cells (Fig. 7B). Interestingly, there was dramatically less colocalization between Pmel17 and LAMP-2 in MART-1-negative WM266-4 cells. The sum of these results indicates that Pmel17 is not trafficked efficiently (but is to some extent) to early melanosomes in the absence of MART-1, as seen in WM266-4 cells.
To validate that this phenomenon is due to the lack of functional MART-1, we then confirmed the colocalization of Pmel17 with various organelles in MART-1-positive cells transfected with mock or MART-1 siRNAs. In these experiments, we used triple staining with secondary antibodies, as described in the figure legend. We selected individual cells that stained for MART-1 using Alexa Fluor 647 to detect MART-1 (Fig. 8, A and B, insets), and we could then readily identify cells in which MART-1 had been successfully knocked down. In MART-1-positive cells, the colocalization of Pmel17 and the ER marker KDEL did not differ visibly between cells transfected with mock or MART-1 siRNAs (Fig. 8A). In contrast, the colocalization of LAMP-2 and Pmel17 was dramatically decreased in cells transfected with MART-1 siRNAs (Fig. 8B). However, a clear correction of Pmel17 trafficking was not observed in WM266-4 cells transfected with MART-1 (Fig. 8B). The sum of these results indicates that the efficiency of Pmel17 trafficking to early melanosomes is significantly interrupted by reducing MART-1 function.

Pmel17 Is Stabilized by MART-1 in the Post-Golgi Compartments—It is clear from the results described above that MART-1 regulates the stability of Pmel17 but an interesting question is in what cellular compartment does MART-1 affect that stability? We first examined that by pulse-chase metabolic

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**Fig. 6. Effects of MART-1 expression on melanosome structure.** WM266-4 cells were examined before (A) and 48 h after (B) transfection with MART-1. Fibrillar melanosomes seen after re-expression of MART-1 are indicated by arrows. Bars, 200 nm.

**Fig. 7. Subcellular distribution of Pmel17.** Cells were fixed and stained with antibodies (αPEP13h, anti-KDEL, and anti-LAMP-2). αPEP13h was detected with Alexa Fluor 594 (red). A, KDEL or B, LAMP-2 antibodies were detected with Alexa Fluor 488 (green). Nuclei were stained with DAPI. Bars, 20 μm.
labeling experiments in the presence of BFA, which traps Pmel17 in the ER. BFA has also been reported to increase the degradation of some substrates in the ER (28). We performed the pulse-chase labeling with Me2SO (as a control), with BFA, with BFA/G132 (benzyloxycarbonyl-Leu-Leu-leucinal, a proteasome inhibitor) and with MG132 alone. The major Pmel17 band appears between P100 and P120 and it increased in size gradually in both types of cells treated with BFA (Fig. 9), although the P26 form became undetectable in the presence of BFA (the latter being consistent with a previous report in MNT-1 cells (3)). The decreased stability of Pmel17 in WM266-4 cells was recovered by treatment with BFA in the presence of BFA (the latter being consistent with a previous report in MNT-1 cells (3)). The decreased stability of Pmel17 in WM266-4 cells was recovered by treatment with BFA in the presence or absence of MG132. Moreover, BFA did not decrease the stability of Pmel17 in SK-MEL-28 cells, which means that BFA did not increase the degradation of Pmel17. The stability of Pmel17 in SK-MEL-28 cells was markedly enhanced by the proteasome inhibitor MG132. MG132 did not similarly prolong the stability of Pmel17 in WM266-4 cells. Interestingly, the kinetics of P26 in WM266-4 cells became similar to that seen in SK-MEL-28 cells. Taken together, the difference in Pmel17 stability between the two cell lines is determined between the post-Golgi and the melanosomal compartments.

DISCUSSION

This is the first report describing the function and role of MART-1 in melanogenesis, showing that it interacts with Pmel17 and regulates its expression, stability, trafficking and processing. The interaction of enzymatic complexes in melanosomes was described by several groups many years ago (29–31) but this is the first report of interactions among structural components of those organelles. We used a multidisciplinary approach to examine the role of MART-1 in melanocytic cells. We adopted siRNA technology to elucidate the function of MART-1 in MART-1-positive melanocytic cells (melanocytes and unpigmented SK-MEL-28 human melanoma cells) as targets. We also used MART-1-negative unpigmented WM266-4 human melanoma cells, with or without re-expression of MART-1, to further clarify the functions of MART-1. We studied the differences between WM266-4 cells and the other types of melanocytic cells to analyze the expression, stability, trafficking, and processing of melanosomal proteins. We used MART-1 siRNA to examine the effects of disrupting MART-1 function and a MART-1 expression vector to examine the effects of increasing MART-1 function.

The P26 form of Pmel17 was identified by immunoblotting...
and by metabolic labeling in MART-1-negative WM266-4 cells, although reactivity with HMB45 was not detected. In other words, Pmel17 processing to the HMB45 detectable form is distinct from its cleavage to the P26 form, although they were previously thought to be related (6). Immunohistochemically, MART-1 accumulates primarily in the Golgi and TGN regions and is only partially localized in early melanosomes (12). This implies that only a small portion of MART-1 is successfully trafficked to early melanosomes, so that even a slight down-regulation of MART-1 disrupts Pmel17 processing to the HMB45 detectable form in melanogenesis (Fig. 4C). In other words, abundant amounts of MART-1 may be required to ensure the correct processing of Pmel17 in early melanosomes. This is also consistent with the observation that re-expression of MART-1 in WM266-4 cells results in the production of fibrillar Stage II melanosomes (Fig. 6). These results suggest that all HMB45-positive melanocytes and melanoma cells should express MART-1 and in fact that is generally the case. In vivo, all HMB45-positive melanomas express MART-1, while 64% of HMB45-negative melanomas also express MART-1 (32). Although the majority of metastatic melanoma cells express both MART-1 and HMB45, 13% express only MART-1 and only 2% of them only express HMB45 (33).

Pmel17 is significantly less stable in WM266-4 cells than in SK-MEL-28 cells, which became prominent in the presence of MG132. Pmel17 (recognized by aPEP13h) is significantly decreased by reducing MART-1 function in the presence or absence of BFA, although the level of Pmel17 mRNA was not affected. Moreover, Pmel17 is slightly up-regulated in WM266-4 cells following the re-expression of MART-1. Immune purification shows that MART-1 and Pmel17 form a complex, and their colocalization was also confirmed by confocal immunohistochemistry. TYRP1 stabilizes TYR function and an enzymatic complex of TYR, TYRP1, and DCT exists in melanosomes (29–31), thus it is reasonable to propose that MART-1 plays a similar role in stabilizing or regulating the structural matrix protein Pmel17.

The stability of Pmel17 in SK-MEL-28 cells was shortened in the absence of functional MART-1. That stability was disrupted in a post-Golgi compartment in the absence of functional MART-1. This is consistent with the result that Pmel17 (recognized by aPEP13h) showed a diffuse band in the 0.8 M sucrose fraction of WM266-4 cells (Fig. 1C). The instability of Pmel17 in WM266-4 cells was observed in the post-Golgi compartment. Pmel17 in WM266-4 cells transfected with MART-1 was stabilized to some extent but did not reach the level of stability seen in SK-MEL-28 cells. Moreover, Pmel17 trafficking to melanosomes in WM266-4 cells was not significantly rescued by transfection with MART-1. This might be because of the relatively low expression level of Pmel17 in WM266-4 cells which might make changes in Pmel17 stability or trafficking difficult to visualize. Interestingly, both sets of results from independent experiments occurred in a post-Golgi compartment. Sorting of Pmel17 in the post-Golgi compartment has not yet been fully characterized. Our recent study showed that some Pmel17 is transported to the cell surface via adaptor protein complex (AP) 3 and is taken up via AP2 while most Pmel17 is directly delivered to Stage 1 melanosomes (34).

MART-1 co-localizes with AP1 but at low frequency and it is possible that it associates with other sorting compartments as well (12). We are continuing to investigate the mechanisms(s) of interaction between Pmel17 and MART-1 in the post-Golgi compartment and the sorting mechanism(s) used by Pmel17 and MART-1 need to be elucidated. Such information will help reveal why Pmel17 trafficking in WM266-4 cells could not be rescued by transfection with MART-1.

MART-1 forms a sufficiently strong complex with Pmel17 to be detected by immunoprecipitation with antibodies to either protein. The MART-1/Pmel17 complex does not require TYR, TYRP1, DCT, or any other melanocyte-specific protein because it could be detected in HeLa cells transfected with only MART-1 and Pmel17. Those complexes are formed in the ER or in the early Golgi as determined by immunoprecipitation studies on subcellular fractions and sequential immunoprecipitation of metabolically labeled proteins. MART-1 modulates Pmel17 function at the post-transcriptional level, affecting its stability, its trafficking to early melanosomes and its processing to the Stage II form. We showed previously that TYR and TYRP1 form a stable complex in melanocytes also within the ER, and we consider two possible interpretations for forming that melanogenic complex, i.e. that binding to other molecules may facilitate their folding and/or that formation of the complex might reduce the cytotoxicity of melanogenic intermediates (21). For Pmel17 at least, formation of the complex with MART-1 might facilitate the expression, stability, trafficking and/or processing of Pmel17. Taken together, these results demonstrate the novel concept that MART-1 is a key regulator for Pmel17 function.

MART-1 has been the most enigmatic melanosomal protein regarding its putative function(s). We have shown in this study that MART-1 forms a complex with Pmel17 and this plays an important role in melanogenesis by regulating the expression,
stability, trafficking and processing of Pmel17, which in turn regulates the maturation of melanosomes. siRNA is a useful tool if a knock-out phenotype is unknown, especially in humans. Future study will be directed to further elucidating the mechanism of MART-1/Pmel17 interactions at the molecular and cellular levels.

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