Characterization and Subcellular Localization of Human Pmel 17/*silver*, a 100-kDa (Pre)Melanosomal Membrane Protein Associated With 5,6,-Dihydroxyindole-2-Carboxylic Acid (DHICA) Converting Activity

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Pmel 17* is preferentially expressed in pigment cells in a manner suggestive of involvement in melanin biosynthesis. The gene is identical to the *silver (si)* pigmentation locus in mice. We now have produced a recombinant glutathione-S-transferase-human Pmel 17 fusion protein and raised polyclonal antibodies against it to confirm the ultrastructural location and presumed site of action predicted by the deduced primary structure of Pmel 17/*silver*, and to authenticate the specificity of the DHICA converting function as inherent to the *silver*-locus protein. Full-length Pmel 17 cDNA also was produced in insect cells in a baculovirus expression vector to ensure that activity did not originate from a co-precipitated protein. Natural hPmel 17 from human melanoma cells has an approximate molecular size of 100 kDa. By immunoperoxidase electron microscopic cytochemistry, the antigen was localized to the limiting membranes of premelanosomes and presumed premelanogenic cytosolic vesicles and, to a minor extent, in the premelanosomal matrix. In an *in vitro* assay, both the natural and the recombinant Pmel 17 accelerated the conversion of DHICA to melanin. This activity was inhibited by the anti-Pmel 17 polyclonal antibodies, indicating that the acceleration of DHICA conversion by the natural protein is genuine and cannot be due to contaminating complexed proteins. We suggest that *in situ* Pmel 17/*silver* is a component of a postulated premelanosomal/melanosomal complex of membrane-bound melanogenic oxidoreductive enzymes and cofactors, in analogy to the electron transfer chain in mitochondria.** ***Key words: melanoma antigen/melanocyte/tyrosinase-related proteins/NK1-beteb/HMB 45.

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Pmel 17, originally isolated from a human foreskin melanocyte λgt11 cDNA expression library, is specifically produced in melanocytes and maps near the *silver* coat color locus on mouse chromosome 10 and to region q12-q13 on human chromosome 12 (Kwon *et al*, 1991).[1] In normal human melanocytes, Pmel 17 mRNA levels correlate with melanin content better than does expression of tyrosinase, the key enzyme of melanogenesis, suggesting that the *silver* protein functions as a positive regulator of melanin biosynthesis distal to tyrosinase (Kwon *et al*, 1987). Like tyrosinase Pmel 17 mRNA expression, in murine Cloudman S-91 and human melanotic melanoma cells is induced by agents that increase melanization (Kwon *et al*, 1987). On the basis of sequence homology, we have proposed that at least two gene families regulate melanin biosynthesis (Kwon, 1993): the tyrosinase gene family, which includes tyrosinase, TRP1/gp75, and DOPAchrome tautomerase or TRP2; and the Pmel 17 gene family, which includes Pmel 17/gp100 (see below) of mammalian melanocytes, MMp115, a chicken melanosomal matrix protein (Mochii *et al*, 1991), and RPE1, a bovine retinal pigment epithelial protein homologous to Pmel 17 (Kim and Wistow, 1992). In its general structure, Pmel 17 is similar to tyrosinase, TRP1, and TRP2 inasmuch as it possesses a putative transmembrane domain and a cytosolic tail (Kwon *et al*, 1991); its amino acid sequence is similar to that of MMp115, with the exception that the latter has no putative transmembrane domain.

Recently, we demonstrated that *Pmel 17* from *si/si* mice contains an (*A*) insertional mutation in the putative cytosolic tail, indicating that Pmel 17 protein is indeed the product of the *silver* locus (Kwon

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Abbreviations: AcNPV, *Autographa california* nuclear polyhedrosis baculovirus; GST-hPmel 17, glutathione-S-transferase-human Pmel 17 fusion protein; *Sf*-21, *Spadoptera frugiperda*-21.

[1] Kim K-K, Heng HHQ, Shi XM, Tsui L-C, Lee ZH, Youn BS, Pickard RT, Kwon BS: Genomic organization and *FISH* mapping of human Pmel 17, the putative *silver* locus. *Pigment Cell Res* (in press).

et al, 1994; Kwon *et al*, 1995). Still in question, however, are the precise subcellular location and function of Pmel 17.

MATERIALS AND METHODS

Production of Human Pmel 17 in Bacteria and Insect Cells For expression in bacteria, a midportion of human Pmel 17 cDNA encoding amino acids 141–435, spanning the repetition motif (Kwon *et al*, 1991), was fused in frame with the glutathione-S-transferase (GST) gene using the pGEX vector (Pharmacia, Piscataway, NJ). The fusion protein GST-human Pmel 17, expressed in *Escherichia coli* strain *Top* 1 (Stratagene, La Jolla, CA), was purified by affinity chromatography over glutathione beads and used to raise polyclonal anti-Pmel 17 antibodies (see below).

A baculovirus expression vector containing the full-size hPmel 17 was constructed for production of a full-length rhPmel 17 in nonmelanogenic insect cells expressing no other melanogenic protein. A 2.0-kb *Eco*R1 fragment of hPmel 17 cDNA encoding the complete protein was inserted into the *Eco*R1 site of the PVL 1393 vector (a gift from Dr. Max Summers, Texas A&M University). The Pmel 17 cDNA was transferred from the PVL 1393-hPmel 17 plasmid to the AcNPV (*Autographa california* nuclear polyhedrosis baculovirus) by cotransfecting both plasmid and virus into *Sf*-21 (*Spadoptera frugiperda*-21) insect cells (a gift from Dr. Max Summers, Texas A&M University), as described (Miller *et al*, 1986). Ten occlusion-negative AcNPV-hPmel 17 viral plaques were isolated and grown as stocks in *Sf*-21 cells in serum-free Ex-Cell 400 medium (JRH Biosciences, Lenexa, KS). The hPmel 17 immunopurified from insect cells was used in functional studies.

Preparation of Rabbit Polyclonal Antibodies and Affinity Purification of Natural and Recombinant Forms of Human Pmel 17 GST-hPmel 17 fusion protein (100 μg) in 0.5 ml Titer-Max (CytRx, Norcross, GA) adjuvant was injected intradermally into each of two rabbits, followed by a second dose 4 weeks later. A booster (500 μg in 0.5 ml phosphate-buffered saline) was given without adjuvant through an ear vein 4 weeks after the second immunization. After a 10-day waiting period, the rabbits were bled, and the IgG fraction was purified on a protein *A* affinity column and stored at −70°C. These antibodies were used in immunopurifications, western immunoblotting, and immunoelectron microscopy.

YU SIT1 melanoma cells, 1×10^9 cells/batch, were lysed in a buffer containing 50 mM Tris HCl, pH 7.6, 300 mM $CaCl_2$, 0.5% Triton X-100, and 10 μg/ml each of proteinase inhibitors chymostatin (Boehringer, Mannheim, Germany), pepstatin, leupeptin, and aprotinin (Sigma Chemical Co., St. Louis, MO). *Sf*-21 cells, infected with AcNPV-Pmel 17 at 0.5 m.o.i. (multiplicity of infection) during a 3-day exposure, were harvested and resuspended in a lysis buffer containing 0.1 M sodium phosphate, pH 6.8, plus 10 μg/ml of each of the above inhibitors.

Extracts from both cell types were centrifuged at 10,000*g* for 10 min before being subjected to immunoaffinity purification. Toward this end, anti-Pmel 17 antibodies were conjugated to Affi-Gel Hz-hydrazide gel (Bio-Rad poly-prep column; Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Pmel 17 was purified from the melanoma and insect cell lysates employing the Immunopure Antigen/Antibody buffer system kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, the supernatants were applied to the columns; nonspecifically bound proteins were removed by exhaustive washing; and specific-binding proteins were eluted with low-pH elution buffer as instructed. Eluted protein solutions were neutralized immediately to pH 7.5 with 1.5 M Tris HCl, pH 8.8, and stored at 4°C.

Western Immunoblotting Two human melanoma cell lines, YU SIT1 and OCM-1, and two nonmelanocytic cell lines, HeLa cells and THP-1 monocytes (ATCC, CCL1, and ATCC TIB202), were used as positive and negative controls for Pmel 17, respectively. The cells were washed in phosphate-buffered saline and lysed for 15 min in ice-cold buffer consisting of 50 mM Tris HCl, pH 8.0, 1% Nonidet P-40, and 1 mM ethylenediamine tetra-acetic acid plus the proteinase inhibitors aprotinin, leupeptin, and pepstatin at 10 μg/ml each. Lysates were centrifuged at 10,000*g* for 10 min at 4°C. The supernatants, containing approximately 1 μg of protein/μl, were denatured by boiling for 2 min in sample buffer.

Cell extracts (10 μg protein/lane), or fractions from anti–Pmel-17 immunoaffinity columns, were subjected to sodium dodecylsulfate (10%)/polyacrylamide gel electrophoresis, transferred onto Immobilon-p membranes (Millipore Corp., Bedford, MA), and incubated with the polyclonal rabbit anti-Pmel 17 antibodies (1:5,000 dilution), followed by alkaline phosphatase–conjugated goat anti-rabbit antibodies and phosphatase chromogenic substrate, according to the manufacturer's instructions (Bio-Rad).

Immunoperoxidase Electron Microscopy The polyclonal antibodies were used at a dilution of 1:250 in the subcellular localization of hPmel 17 antigen. Amelanotic melanocytes from a tyrosinase-positive adult human albino were grown in melanocyte medium as described (Halaban *et al*, 1988). Monolayers were fixed *in situ* in a cold mixture of 1% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). During subsequent washing (5 min), unreacted aldehyde was neutralized with 0.1% glycine in phosphate-buffered saline. Cells were then permeabilized with 0.02% Triton X-100 (1 min), and nonspecific binding sites were blocked with commercial serum (anti-rabbit *Vectastain ABC* kit; Vector Laboratories, Burlingame, CA) (30 min). The remaining steps of the immunocytochemical procedure were according to the *Vectastain* immunoperoxidase kit instructions. In the hope of selectively inactivating intrinsic catalase (peroxidase), we exposed some preparations briefly to 0.03% H_2O_2 before incubating them with the anti-Pmel 17 antibodies. Before dehydration and embedding in Epoxy resin, the cells were refixed with cacodylate-buffered 3% glutaraldehyde, followed by a mixture of buffered 1% OsO_4/1.5% potassium ferrocyanide for contrast. Ultrathin sections, cut in a plane parallel to the culture surface, were counterstained with lead for further contrast and viewed in a ZEISS EM 109 transmission electron microscope.

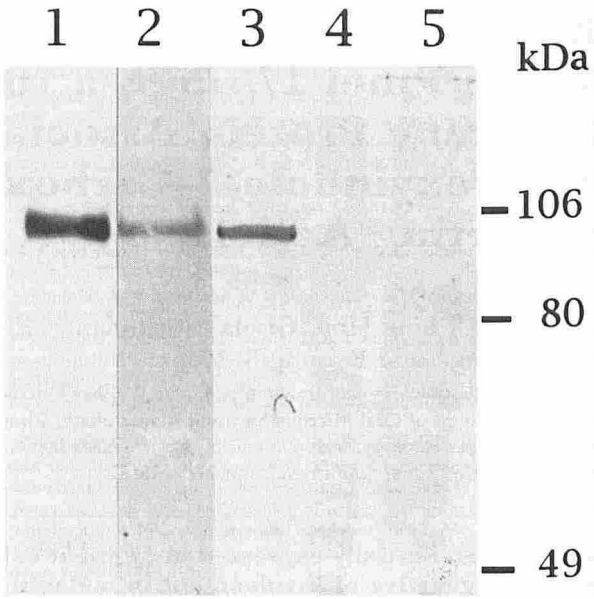


Figure 1. Western immunoblots of hPmel 17/100-kDa protein. *Lane 1*, whole-cell lysate of YU SIT1 metastatic melanoma; *lane 2*, immunoaffinity-purified Pmel 17 (1 ng protein) from YU SIT1 lysate; *lane 3*, whole-cell lysate of OCM-1 ocular melanoma; *lanes 4* and *5*, whole-cell lysates of nonmelanocytic control cell lines THP-1 and HeLa. Whole-cell lysates were applied at 10 μg protein/lane.

DHICA Conversion Assay The DHICA conversion assay was performed as follows. Reaction mixtures (final volume 550 μl) consisted of 100 μl affinity-purified hPmel 17 or rhPmel 17 (0.1 μg/ml), 5 μl anti-Pmel 17 IgG (10 μg/μl) where applicable, 350 μl buffer containing 0.1 M sodium phosphate and 0.1 mM phenylthiourea, pH 7.0, plus 100 μl of 0.5 mM DHICA (a gift from Drs. Ashok Chakraborty and John Pawelek, Yale University). The mixtures were incubated at 37°C for various periods of time, at the end of which the optical density shift due to melanin formation was measured in a Beckman DU-64 spectrophotometer set at optical density 400 nm.

RESULTS

Size of Human Pmel 17 We had shown previously that the hPmel 17 gene was expressed preferentially in pigment cells. The amino acid backbone deduced from the cloned cDNA was 68.6 kDa. In the present study, expression and molecular size were determined by Western immunoblotting **(Fig 1)**. Pmel 17 appeared as approximately a 100-kDa protein in whole-cell lysates from two human melanoma cell lines and in several immunoaffinity-purified fractions. The higher apparent molecular weight of the native protein can be attributed to glycosylation. The recombinant hPmel 17 protein expressed in insect cells was somewhat smaller than the mammalian pigment cell product (data not shown). No Pmel 17

was detected in nonpigment cells, such as HeLa cells or THP-1 monocytes. Some affinity-purified fractions were resolved as two reactive bands: the 100-kDa band and another of 52 kDa (data not shown). The smaller protein could represent a degradation product of Pmel 17, but more likely is anti-Pmel 17 IgG heavy chain leached from the column.

Subcellular Localization of Human Pmel 17 The abundant distribution of Pmel 17 gene product in human melanocytes is illustrated in **Fig 2**. We used tyrosinase-positive albino melanocytes to avoid interference by melanin with both the accuracy of localization and the clarity of visualization of the immunoperoxidase reaction product. The ultrastructure of the amelanotic melanocytes in a control preparation that was incubated without the primary antibodies is shown in **Fig 2*a***. The cells contained well-formed premelanosomes of the eumelanosome type, with limiting membranes devoid of peroxidatic reaction product or melanin. The myriad small electron-dense cytoplasmic vesicles may be microperoxisomes, with peroxidase activity due to catalase (Novikoff *et al*, 1973). Vesicles of the same size in controls not incubated for peroxidase activity were devoid of such contrast (not illustrated). Some of the microperoxidatic vesicles had been internalized by premelanosomes or were present in multivesiculate bodies.

Bound polyclonal anti-Pmel 17 antibodies, on the other hand **(Fig 2*b*)**, were localized to the limiting membranes of the (pre-) melanosomes and to cytoplasmic vesicles, many of which were of the coated variety and larger than the presumed microperoxisomes. The unreactive membranes of mitochondria and endoplasmic reticulum served as built-in controls.

The reaction product was located at the internal surface of the (pre)melanosomal and vesicular membranes **(Fig 2*c*)**, consistent with an epitope or epitopes along the *N*-terminal extracytosolic extension of Pmel 17, which contains the presumptive glycosylation sites. Again, a few (pre)melanosomes also contained reaction product in the form of globules within the matrix. Preincubation with H_2O_2, in an attempt to inhibit selectively native catalase, markedly diminished antibody binding but underscored the membrane and matrix-globular localizations **(Fig 2*d*)**.

DHICA Converting Activity of Pmel 17 Because Pmel 17 from pigment cells may be complexed with other melanogenic proteins (Orlow *et al*, 1994) and during immunopurification may tag along tyrosinase, TRP1, and TRP2, and perhaps others not yet known, recombinant hPmel 17 expressed in the nonmelanogenic *Sf*-21 insect cells was immunopurified and tested for DHICA-to-melanin converting activity. In contrast to parental cells or cells infected with an irrelevant recombinant baculovirus, the immunopurified baculovirus-expressed Pmel 17 tested positive **(Fig 3*a*)**. This activity was protein dose dependent. Furthermore, fractions containing immunoaffinity-purified native hPmel 17, as identified by western blot analysis **(Fig 1)**, were pooled and assayed for DHICA-to-melanin converting activity in the presence and absence of anti-Pmel 17 antibodies. The DHICA converting activity was blocked by the antibodies **(Fig 3*b*)**.

DISCUSSION

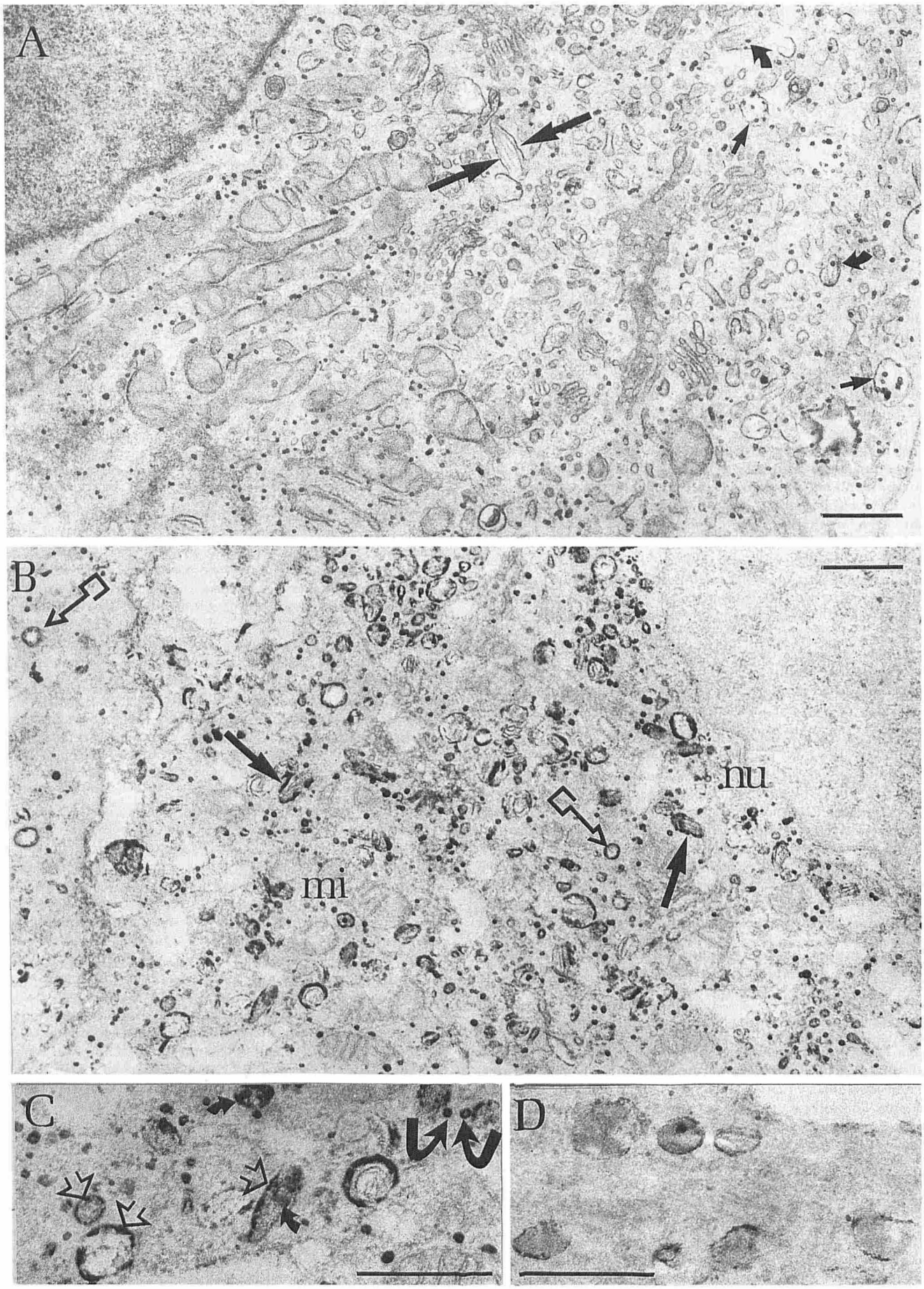
We have shown that human Pmel 17/*silver*, a protein of 100 kDa, is located in vesicular and (pre)melanosomal limiting membranes and that *in vitro*, this protein accelerates the conversion of DHICA to melanin. The enzymatic activity was detected in immunoprecipitates of melanoma cell extracts using polyclonal anti-Pmel 17 antibodies and in recombinant Pmel 17 produced in nonmelanogenic insect cells. The latter ensured that the DHICA conversion activity did not originate from a co-precipitated melanogenic protein. Our data complement those of Chakraborty *et al*,[2] who reported superoxide-dependent polymerization of DHICA in the presence of immunopurified native Pmel 17. Because it facilitates the formation of melanin and is located in the limiting membranes of known melanogenic subcellular organelles, Pmel 17 may be a constituent of a postulated membrane-bound melanogenic complex of oxidoreductive enzymes (Orlow *et al*, 1994) that include tyrosinase (*Albino* or *c*-locus protein) and the tyrosinase-related proteins TRP1 (gp75/*b*-locus protein [Jackson, 1988; Halaban and Moellmann, 1990; Tomita *et al*, 1991]) and TRP2 (DOPAchrome tautomerase/*slaty*-locus protein [Krompouzos *et al*, 1994]), in analogy to the electron transfer chain in mitochondria.[3] Matrix components bound the anti-hPmel 17 antibodies in a distribution consistent with vesiculoglobular bodies (Jimbow and Fitzpatrick, 1994).

The membrane localizations confirm predictions based on the deduced amino acid sequence of Pmel 17 (Kwon *et al*, 1991). This sequence calls for a glycoprotein with a transmembrane domain and a cytosolic tail, with the majority of the glycosylated chain extending into an extracytosolic space, such as a vesicular lumen (e.g., melanosome), or, in the case of vesicular exocytosis, an extracellular space where the protein may become antigenic. In fact, a protein analogous to Pmel 17, referred to as gp100 (Adema *et al*, 1994) and recognized by monoclonal antibodies NK1/beteb, HMB-45, and HMB-50 (Schaumburg-Lever *et al*, 1991; Adema *et al*, 1993; Taatjes *et al*, 1993), has been identified as a potent tumor-derived antigen in melanoma patients of the human leukocyte antigen–A2 haplotype (Cox *et al*, 1994; Kawakami *et al*, 1994). Pmel 17 and gp100 are translation products of alternatively spliced mRNA (Adema *et al*, 1994). The membrane localization of Pmel 17 agrees with the binding sites of NK1/beteb (Winder *et al*, 1994), an antibody raised against a membrane preparation from a human metastatic melanoma (Vennegoor *et al*, 1988), and with the immunogold data obtained with rabbit polyclonal antibodies raised against a melanosomal fraction from murine B16 melanoma cells (Orlow *et al*, 1993b; Zhou *et al*, 1994). The antigenic sites were localized to the extracytosolic domain of the membranes, consistent with an epitope on the postulated intramelanosomal glycosylated *N*-terminal extension of Pmel 17. In all cases, the orientation was identical to one obtained with anti-PEP2, an antiserum specific for the predicted luminal portion of the melanosomal membrane protein TRP1 (Orlow *et al*, 1993). Our data, as supported by others, including the immunogold data of Orlow *et al* (1993b), are consistent with the *silver*-locus product being a *bona fide* membrane protein.

The importance of Pmel 17 to pigmentation is indicated from the changes in melanocyte survival in mice carrying the *si/si* (*silver*) mutation (Quevedo *et al*, 1981). The single nucleotide insertion is expected to alter the last 24 amino acids of the original *C*-terminus and lengthen the protein by 12 residues. These changes are likely to misguide the mutant protein away from melanogenic membranes because they bring about the loss of a melanosomal targeting sequence located in the *C*-terminal extension of Pmel 17 and other melanogenic membrane proteins (Vijayasaradhi *et al*, 1995). The premature death of follicular melanocytes in the *silver* mice suggests that Pmel-17–accelerated conversion of DHICA to melanin is required for the speedy removal of cytotoxic intermediates deriving from earlier oxidation-reduction reactions in the melanogenic pathway. Genetic support for this notion comes from the observation that in *Agouti/silver* and in *Agouti/yellow/silver* mice, silvering decreases rather than increases with age (Silvers, 1979). *Agouti* and *Agouti/yellow* mice suffer from a mutation that results in a blockade of the melanocytic receptor for melanocyte-stimulating hormone (melanotropin) (Lu *et al*, 1994), thereby decreasing expression of tyrosinase (Halaban *et al*, 1984) and Pmel 17 (Kwon *et al*, 1987). It is reasonable to postulate that in the above mutants, with a constitutional decrease in eumelanogenic cytotoxic intermediates, a

[2] Chakraborty AK, Platt JT, Kim K-K, Kwon BS, Bennett DC, Pawelek JM: Polymerization of 5,6-dihydroxyindole-2-carboxylic acid to melanin by the Pmel 17/silver locus protein. *European J Biochem*, 1996, *in press*.

[3] As suggested by Moellmann, XIV International Pigment Cell Conference, Kobe, Japan, 1990.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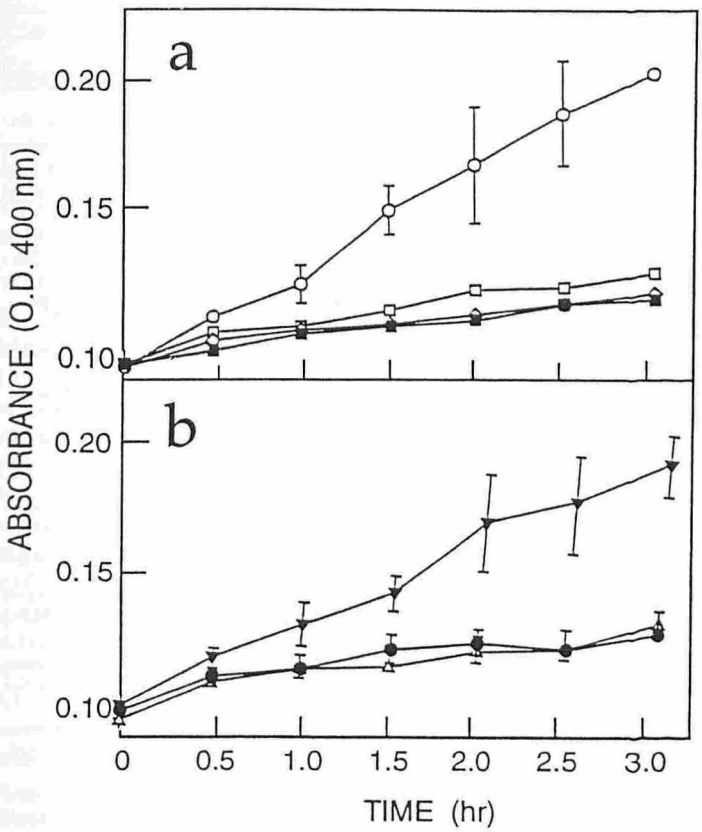

Figure 3. Facilitation of conversion of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into soluble melanin by recombinant as well as native human Pmel 17. a) DHICA converting activity of immunopurified recombinant human Pmel 17 (rhPmel 17; ○), whose synthesis was directed by a recombinant baculovirus in *Sf*-21 insect cells; identical columns and procedures were used with control wild-type virus-infected *Sf*-21 cells (□) and *Sf*-21 cells infected with an unrelated recombinant baculovirus (■). *Diamonds*, buffer alone. b) DHICA converting activity in immunopurified native hPmel 17 from YU SIT1 melanoma cells. ▲, hPmel 17; ●, hPmel 17 plus anti-hPmel 17 antibodies; △, buffer alone. Concentrations as in *Materials and Methods*. Each point represents mean ± SD.

superimposed *silver* mutation is not as melanocytotoxic as in eumelanotic, wild-type mice.

The *silver* mutation has long been known to affect coat color dilution, most noticeably when expressed on a *B/b* (genetic locus encoding TRP1) background (Silvers, 1979). Pmel 17 may, indeed, complement most strongly with TRP1, the melanosomal membrane protein that appears to have both DHICA synthetic activity (dopachrome tautomerase [Winder *et al*, 1993, 1994]) and its own DHICA converting activity (DHICA oxidase [Johnson and Jackson, 1992; Jiménez-Cervantes *et al*, 1994; Kobayashi *et al*, 1994]), as well as being associated with catalase activity (Halaban and Moellmann, 1990). The genetically dominant mouse TRP1 mutation *Light* (B^{lt}) causes premature melanocyte death in a phenotypic pattern similar to that in *si/si* mice (Quevedo *et al*, 1981). The B^{lt} mutation consists of a single amino acid substitution near the *N*-terminus of TRP1, close to the putative signal-peptide cleavage site (Johnson and Jackson, 1992), and may adversely affect the insertion of the nascent protein into the endoplasmic reticulum, and hence melanosomes. The genetic evidence that two melanosomal proteins, which for different reasons may have difficulty entering premelanosomes, produce similar phenotypes strengthens the conclusion that both have similar functions. Nevertheless, the relative importance of Pmel 17 is underscored by the findings of Quevedo and Chase (1958), who showed that through repetitive induction of the hair cycle by plucking and replucking, the follicular melanocyte population is rapidly depleted in *si/si* but not B^{lt}/B^{lt} mice.

Extrapolating from what is known about the murine *silver* locus, we suggest that human Pmel 17 may have a role in early graying and/or postnatally acquired white spotting (vitiligo), conditions in which melanocytes die. A search for a Pmel 17 mutation, not necessarily identical to that in the silver mouse, may be indicated in afflicted individuals.

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Figure 2. Subcellular localization of hPmel 17 protein to premelanosomal and presumptive premelanogenic vesicular membranes. *A*, control incubation (anti-Pmel 17 antibodies omitted). The premelanosomes and their limiting membranes are devoid of immunoperoxidase reaction product (example between *large arrows*). The myriad small electron-dense vesicles may be microperoxisomes containing native peroxidatic activity due to catalase. Some of these vesicles have been internalized by premelanosomes (*small curved arrows*) and multivesicular bodies (*small curved arrows*). *B*, immunoreactive preparation. Anti-Pmel 17–immunoperoxidase product outlines the limiting membranes of premelanosomes (*large arrows*) and premelanogenic vesicles (examples at *angular arrows*). The latter are larger than the presumed microperoxisomes. *mi*, mitochondria; *nu*, nuclear membrane. *C*, detail from immunoreactive preparation *B*. The anti–Pmel-17 immunoperoxidase product is found on the extracytoplasmic (internal) surface of (pre)melanosomal limiting membranes and on the membranes of presumed premelanogenic vesicles (*open arrows*). Some of the reactive premelanosomes have peroxidase-positive vesicles within their matrix (*small curved arrows*); others appear as being approached by cytosolic vesicles (*large bent arrows*). *D*, antibody binding was diminished by preincubation of the cells with H_2O_2 (to inactivate intrinsic peroxidase due to catalase), but the localization of anti-Pmel 17 to (pre)melanosomal membranes and vesiculoglobular bodies persisted. *Scale bars*, 0.5 μm.

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