The silver mutation in mice causes progressive graying of hair due to the loss of functional follicular melanocytes. The silver locus gene (called Pmel 17) has been cloned; its encoded product shares homology with a chick melanosomal matrix protein and a bovine retinal pigment epithelial protein. Although the sequence of the silver gene and the correlation of its expression with pigment production have been reported, its function in melanogenesis is still unknown. In an effort to characterize that function, we have synthesized the predicted carboxy-terminal peptide of the mouse Pmel 17 protein and generated a rabbit polyclonal antibody (aPEP13) to it; that antibody recognized the silver protein specifically. The immunoaffinity-purified silver protein lacked all of the known melanogenic catalytic activities which other tyrosinase-related proteins (TRP) have, nor did it appear to modulate any of those TRP activities. Metabolic labeling experiments demonstrated that the silver protein disappears in vivo within a few hours, indicating that it is rapidly degraded, or quickly processed to lose its carboxyl terminus. Cross-reactivity experiments showed that a recently reported antigelatinase matrix metalloproteinase antibody (a2M) also recognizes the silver protein, although at a diferent epitope from that of aPEP13. Using Western immunoblotting, we analyzed subcellular fractions isolated from B16 F10 melanoma cells and found that the silver protein was rich in the melanosomal fraction but was absent from coated vesicles which deliver TRPs to melanosomes. These results suggest that the silver locus product is a melanosomal matrix protein which may contribute to melanogenesis as a structural protein, although the possibility remains that it also has a novel catalytic function in melanogenesis.

The murine silver locus is one of more than 50 loci which control coat color, and the recessive silver mutation (si) causes a progressive loss of coat hair pigmentation (1). Like the three members of the tyrosinase-related gene family of melanosomal proteins, the silver gene is thought to act at the subcellular level (2). Recently a gene, named Pmel 17, was cloned and mapped to the silver locus (3, 4). The expression of Pmel 17 mRNA was shown to be melanocyte-specific and both β-melanotropin and isobutylmethylxanthine could stimulate its expression concurrently with increases in melanin synthesis (5). The predicted amino acid sequence of the product of the silver locus (termed the silver protein in this report) has potential glycosylation sites and a putative transmembrane region close to its carboxyl terminus; the predicted molecular mass of the protein backbone is about 70 kDa, which is very similar to the tyrosinase-related proteins (TRPs). Further, the silver protein has a unique high content of serine and threonine, and three repeats of a 26-amino acid motif in the middle of its sequence (3). The recently identified melanosomal matrix protein MMP115 of chicken retinal pigment cells (6) and the bovine retinal pigment epithelial protein RPE-1 (7) share significant sequence homology with the silver protein (8).

However, the function of the silver protein in mammalian melanogenesis remains unclear. The silver mutation has been reported to result in the graying of coat hairs by causing the premature loss of functional melanocytes in hair follicles (9, 10). The slower growth of silver mutant melanocytes (melan-si) in culture compared to wild type melanocytes has also been observed (11). Therefore, the silver mutation seems likely to induce toxic effects to melanocytes analogous to those caused by the phenotypically similar Bk mutation at the brown locus (10, 12). Melanocytes are continually exposed to the stress of potentially toxic intermediates of melanogenesis, particularly 5,6-dihydroxindole (DH) (13). The silver protein might protect melanocytes against such intermediates through a catalytic function (e.g. by their rapid removal or selective modulation of their production), or, alternatively, it might function as a structural melanosomal matrix protein restricting melanogenesis to that subcellular compartment.

To characterize the silver protein, and to determine its function, we have prepared a rabbit polyclonal antibody (termed aPEP13) against the synthetic peptide corresponding to the mouse Pmel 17 carboxy terminus (14), a technique we have previously used to characterize the TRPs encoded by the albinos, brown, and slaty loci (15–18). In a recent collaborative study (19), we found that an antibody (a2M) generated against melanosomal matrix components recognized a protein encoded at the silver locus, although additional melanosomal matrix proteins are also recognized by the a2M antiserum. In this
study, we show that aPEP13 specifically recognizes the silver protein, and we have used it to immunopurify the silver protein for analysis of possible catalytic functions and to examine its synthesis, processing and subcellular distribution.

**Experimental Procedures**

**Cells and Culture Conditions**—B16 F10 murine melanoma cells were cultured as described previously (16, 17); the B16 F10 subline used in this study was virtually amelanotic but could be induced by α-melanotropin to increase production of tyrosinase and melanin (20). Mela-

nocye cell lines cultured from genetically defined mice (melan-a, melan-b, melan-c) were grown as described previously (16, 17, 20). Briefly, semiconflu-

ent cells were preincubated in methionine-free medium containing dia-

lyzed fetal bovine serum, pulsed for 30 min at 4 °C with [35S]methionine (0.4—1.0 μCi/ml) (Du Pont-NEN) in methionine-free medium, andchase in complete medium for 0—48 h, as detailed in the figure legends. The cells were harvested with trypsin/EDTA, washed with phosphate-

buffered saline without CaCl2 and MgCl2 (PBS) and solubilized at 4 °C for 60 min in Nonidet P-40/SDS buffer (1% Nonidet-P40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, 100 μg phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin). The resulting [35S]-labeled extracts were then preincubated with 10 μl of normal rabbit serum and 100 μl of Gammabind G Sepharose (Pharmacia Biotech Inc.). 2 × 106 trilocholic acid-precipitable counts/min of precleared extracts were incubated with 5 μl of antibodies for 1 h at 4 °C, and then complexed with 30 μl of Gammabind G Sepharose for 30 min at 4 °C. The immune complexes were washed six times with Nonidet P-40/SDS buffer, then eluted in SDS sample buffer containing 100 μM diethiothiol at 95 °C for 5 min and analyzed by SDS-gel electrophoresis (24), followed by fluorography.

**Western Immunoblotting Analysis**—Proteins from Nonidet P-40/SDS solubilized cells or tumors, or immunoprecipitated proteins as detailed below, were separated on 10% SDS gels, blotted onto polyvinylidene difluoride membranes (Immo-

bilon-P, Millipore Corp., Bedford, MA) and incubated with primary anti-

bodies (1:100 dilution) as noted in the figure legends. Subsequent visualization of antibody binding was carried out with Enhanced Chemiluminescence (Amersham Corp.) according to the manufacturer's instructions.

**Immuno-affinity Purification**—Immuno-affinity columns were pre-

pared by covalently linking 1 mg of purified anti-peptide IgG to 2 ml of protein A-Sepharose columns using IgG Orientation Kits (Pierce), ac-

cording to the manufacturer's instructions and as detailed elsewhere (16—18). B16 F10 melanoma cells growing spontaneously in mice were ex
cised, homogenized and layered in 155 mM NaCl, 10 mM KCl, 0.1 M EDTA, and centrifuged for 40 min at 10,000 × g. The pellets were then extracted with 50 mM phosphate buffer, pH 6.8, 0.1 mM EDTA, 10 μg phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin overnight at 4 °C. The supernatants were divided into 2 ml portions and incubated with 20 μl of aPEP13 IgG or control IgG. After an overnight incubation at 4 °C, the IgG complex was precipitated by the addition of 4 ml of a 1:1 mixture of cold Tris-buffered saline and ethanol. The pellets were washed twice with cold Tris-buffered saline and once with 1× PBS. The eluted fractions containing the silver protein were then analyzed by Western immunoblotting, collected, dialyzed and concentrated by freeze-drying.

**Isolation of Melanosomes and Coated Vesicles**—Isolation of melano-

somes was carried out as described previously (25). All steps were per-

formed at 4 °C. B16 F10 melanoma cells were washed twice with PBS, centrifuged for 10 min in homogenization buffer (0.25 mM sucrose, 50 mM phosphate buffer, pH 6.8, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), homogenized with twelve strokes of a Potter-Elvehjem grinder. Following centrifugation for 10 min at 1000 × g, the supernatant was centrifuged for 30 min at 45,000 × g. The pellet was resuspended in homogenization buffer and applied to a discontinuous gradient of 1.0—2.0 M sucrose and centrifuged for 1.5 h at 100,000 × g. Melanosome-rich fractions were collected at the 1.8—2.0 x interfaces and washed three times with PBS.

**Isolation of coated vesicles was performed as reported previously (26). B16 F10 melanoma cells were isolated, lysed, washed with ice-cold phosphate-buffered saline, and mixed with an equal volume of MES buffer (0.1 M MES-NaOH, pH 6.5, 1 mM EDTA, 0.5 mM MgCl2, 0.02% NaN3, 100 μM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). The mixture was homogenized with a Potter-Elvehjem grinder and centrifuged for 40 min at 19,000 × g. The supernatant was then dialyzed in MES buffer, then mixed with equal volume of 12.5% sucrose and 12.5% Ficoll in MES buffer, and centrifuged again for 70 min at 43,000 × g. The super-

nats were diluted in MES buffer, centrifuged again at 43,000 × g, and coated vesicles were collected in the pellet.

**Molecular Characterization of aPEP13**—The eluted silver protein catalytic activities as described below were carried out at pH 6.8, 37 °C for 60 min unless otherwise noted in the text. 1) Tyrosine hydroxylase activity was measured using the [3H]tyrosine assay (27, 28). This method specifically measures the brain tissue water produced during the hydroxylation of ty-

rosine to DOPA. DOPA oxidase activity was measured using incorpora-

tion of [3-3H]DOPA, an acid-insoluble melanin obtained previously (20). DOPAchrome tautomerase activity was measured by high performance liquid chromatography as the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than DHI; data are converted to picomoles of DHICA produced. 3) DOPAchrome tautomerase activity was measured by high performance liquid chromatography as the disappearance of DHI substrate from reaction mixtures compared to controls for spontaneous auto-oxidation; data are converted to pmol by comparison with known standards. 4) DHI oxidase activity was measured by high performance liquid chromatography as the disappearance of DHI substrate from reaction mixtures compared to controls for spontaneous auto-oxidation; data are converted to pmol by comparison with known standards. 6) Melanin production was measured using incorporation of [3H]tyrosine into acid-insoluble melanin as detailed previously (27, 28); picomoles of melanin produced are calculated from the radioactive product. Tyrosine and DOPA used as standards and reaction substrates in these assays were obtained from Sigma; [2-3H]DOPA, [3-3H]tyrosine, and [3-3H]tyrosine DOPA oxidase were obtained from Du Pont-NEN; DOPAchrome was prepared using the silver oxide method originally described by Körner and Pawelek (30). DHI and DHICA were kindly provided by Dr. Giuseppe Prota (University of Naples, Naples) and purchased commercially from Regis Chemical Co. (Morton Grove, IL).

**Miscellaneous Methods**—Protein concentrations were determined with the BCA assay kit (Pierce) using bovine serum albumin as a standard.

**Results**

**Preparation and Specificity of Antibody to the Silver Protein**—We synthesized the 15 amino acid peptide (named aPEP13), which corresponds to the carbonyl terminus of the predicted protein encoded by the murine Pmel 17 gene (14), which has been mapped to the silver locus (3, 4). The rabbit polyclonal antibody termed aPEP13, generated against PEP13 and cross-reactive to another peptide encoded by the murine Pmel 17 gene (14), was shown by enzyme-linked immunosorbent assay (15). Each of the antibodies was immunizing peptide specific and cPEP13 IgG had almost the same titer against the immunizing peptide as the other antibodies had. Note that aPEP1, aPEP7, and aPEP8 did not react with PEP13, nor did the aPEP13 recognize peptide PEP1, PEP7 or PEP8; aPEP1, aPEP7, and aPEP8 recognize distinct melanogenic proteins.
peptides as noted were bound to 96-well Immulon I1 plates, antisera buffered saline, and the binding of antibodies was measured by enzyme-linked immunosorbent assay as described under "Experimental Procedures." The data reported are the average A405 of duplicate wells.

Although aPEP13 reacted with several minor low molecular weight bands on the immunoblot filter, it recognized the major band only in the extracts of normal and transformed melanocytes, at the same molecular size as in the metabolic labeling study (Fig. 2A). The observed molecular mass of the protein identified by aPEP13, 85 kDa, was somewhat larger than that expected from the amino acid sequence predicted for human Pmel 17 (i.e. 70 kDa). This was a single band, very sharp and thin as compared with the broadly migrating ones of the TRPs. Fig. 2, A and B, also reveal that the highly pigmented melan-a melanocytes expressed more silver protein than did the unpigmented B16 F10 melanoma cells, consistent with the previous report correlating Pmel 17 expression with pigmentation (6).

We used aPEP13 IgG in immunoprecipitation analysis of [35S]methionine-labeled extracts of NIH 3T3 cells (murine fibroblasts), Meth A cells (murine sarcoma cells), B16 F10 cells (unpigmented murine melanoma cells), and melan-a cells (highly pigmented immortalized murine melanocytes) (Fig. 2A). As with the TRP family, the protein precipitated by aPEP13 was observed only in cells of melanocytic origin (i.e. B16 F10 and melan-a). By Western immunoblotting analysis, we observed a similar pattern of cell-specific expression of aPEP13 antigen and the other melanogenic proteins (Fig. 2B).

It has been reported that the silver mutation is the result of a single base insertion which alters the predicted carboxyl terminus sequence of the protein (14). The mutated silver protein would therefore not be expected to be recognized by aPEP13, whose immunizing peptide was at the carboxyl terminus. When we examined cell extracts of melan-si cells (derived from mice homozygous for the silver mutation) by Western immunoblotting, the 85-kDa protein identified by aPEP13 was restricted to the melanogenic protein (skin) where tyrosinase activity (tyrosine hydroxylase and melanin formation activity) could also be detected (data not shown).

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These results, combined with the cell- and organ-specific expression, and lack of reactivity to the mutated protein, clearly demonstrate that aPEP13 identified the protein encoded by the silver locus.

Immuno-affinity Purification of the Silver Protein and Enzymatic Analysis—Since there was a good correlation of silver gene expression with visible pigmentation and since the silver mutation may result in toxicity to melanocytes, the silver protein has been anticipated to have some specific catalytic function(s) comparable to the other TRPs, or alternatively, an inhibitory effect on the production of harmful intermediates produced during melanogenesis. In order to determine if the protein encoded by the silver locus has any known melanogenic catalytic function, we purified the silver protein from B16 F10 tumors using immuno-affinity chromatography and then examined its potential melanogenic activity. TRPs and the silver protein binding to immuno-affinity columns were eluted, dialyzed and concentrated as detailed in the experimental procedure section and their purities were analyzed by Western immunoblotting. The fraction immuno-purified by the aPEP13 IgG-linked column was recognized only by aPEP13 without any cross-reactivity to other anti-peptide antibodies (Fig. 4). The immunopurified protein had the identical size described in the radiolabeling experiments above. The other low molecular weight band (arrow) was observed in all blots and all fractions, and is IgG eluted from the columns. Western immunoblotting
Characterization of the Silver Locus Protein

Melanogenic assays of these purified proteins demonstrated that the silver protein had no known catalytic function attributed to the members of the TRP family (Table I). As previously reported (17, 18), the TRPs each have specific enzymatic function(s): tyrosine hydroxylase, DOPA oxidase, DHI oxidase and melanin formation activities of tyrosinase; DHICA oxidase activity of TRP1; DOPAchrome tautomerase activity of TRP2 (Table I). TRP2 had detectable levels of tyrosinase catalytic activities, which were consistent with the slight contamination of this immunopurified fraction with tyrosinase. The immunopurified silver protein, however, had just the baseline, negative control level of each of those melanogenic activities. Although the purified silver protein appears to have a slight DHI oxidase activity, this is not statistically significant above background.

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Characterization and Subcellular Fractionation of the Silver Protein—The immunopurification experiments reported above suggested that the silver protein does not have a demonstrable known melanogenic enzymatic function. In further studies, we examined how it is synthesized, processed and degraded, and where it is delivered and exists within the melanocyte.

It is well known that tyrosinase, TRP1 and TRP2 are highly glycosylated proteins (31–33). When melan-a or melan-si cells were pulse-labeled by [35S]methionine and then chased, the presence of inhibitors of glycoprotein processing (swainsonine or deoxymannojirimycin) decreased the molecular weight of TRPs, from those of the glycosylated forms seen in the normal chase for 1 h (Fig. 5), as has been reported previously (31). However, identical treatments had no effect on the size of band precipitated by aPEP13 (Fig. 5). The immunoprecipitated band of the silver protein also was not affected when the labeled cell extract was treated with neuraminidase under conditions in which TRP1 was shifted to the de novo type completely (data not shown). These data demonstrate that the silver locus product is not subject to late Golgi processing of Asn-linked carbohydrates although it has several potential sites for N-glycosylation (3).

When the 30-min pulse labeling of cells was followed by longer chases in the unlabeled medium and immunoprecipitation analysis, the TRPs were observed to be fully glycosylated from the de novo type and then degraded (Fig. 6, left), as previously reported by our group (17). The silver protein, however, was not similarly processed to a higher molecular weight form and disappeared completely within 4 h. Similar results were obtained with B16 F10 melanoma cells. A Western immunoblotting experiment (Fig. 6, right) shows that majority of the silver protein in cell lysates could be recognized by aPEP13 even after incubation for 48 h at 37 °C. These results indicate that the silver protein is inherently stable (as are the TRPs) but that it is actively processed to lose its carboxyl terminus (the aPEP13 epitope) within a few hours in vivo within melanocytes. When the 35S-labeled and 4-h chased cell extracts were treated by neuraminidase (conditions, under which the molecular weight of immunoprecipitated TRPs are decreased) or were denatured in 1% SDS, 20 mM DTT, aPEP13 still could not precipitate a band from those extracts (data not shown).

Since these data suggest that the silver protein must have lost its carboxyl terminus during post-translational processing, we tested other antibodies which might recognize the silver protein at an epitope different from aPEP13. We found that aMX, which has been recently described (34), also recognized the silver protein along with other or yet unidentified matrix constituents (19). That study showed that, when 35S-labeled cell extracts were preclued with either aMX or aPEP13, reactivity with the other antibody was lost. We now report that the specificity of aMX was identical to that of aPEP13; the silver protein recognized by aMX is specific to transformed and normal melanocytes (Fig. 7, A and B). However, aMX reacted with the silver protein of melan-si cells by Western immunoblotting (Fig. 7B), suggesting that aMX recognizes epitope(s) distinct from aPEP13. To map the epitope recognized by aMX on the silver protein, we used partial digestion with V8 protease, as described previously (16, 35). We purified the silver protein by preparative electrophoresis, digested it with V8 protease, separated the fragments by SDS-polyacrylamide gel electrophoresis, and analyzed the reactivity of aPEP13 or aMX by Western blotting (data not shown). aPEP13 reacted with a 25-kDa fragment generated by digestion of the silver protein with V8 protease whereas aMX did not recognize that fragment, indicating that aMX recognizes an epitope of the silver protein at least 25 kDa apart from its carboxyl terminus.

Orlow and collaborators (34) have found, using pulse-chase metabolic labeling, that an the melanosomal matrix protein recognized by aMX disappeared, a band of 53 kDa appeared after long chases. That result was reproduced in this study, but since aMX was generated against a mixture of melanosomal matrix proteins extracted in SDS buffer, it was possible that the 53-kDa band was a separate, unrelated protein, and not a processed form of the silver protein. To examine this possibility, we tested whether the recognition of the 53-kDa protein by aMX could be removed by incubation with the silver protein. Although the purified silver protein had the specific ability to compete with the binding of aPEP13 to its ligand and of aMX to the 85-kDa band, it had no similar ability to compete with binding of aMX to the 53-kDa band (Fig. 8). Therefore we conclude that the silver protein was not processed to the 53-kDa form.

Based on the cross-reactivity experiments of aPEP13 and...
Figure 4. Western immunoblotting analysis of immune-purified fractions. Silver protein, tyrosinase, TRP1, and TRP2 were immune-affinity-purified from B16 F10 melanoma cells as described under "Experimental Procedures." Fractions were separated by SDS-gel electrophoresis, transferred to nitrocellulose membranes, and examined for reactivity with antibodies as noted for Fig. 2B. P1, purified through αPEP1 immunofinity column (TRP1); P7, purified through αPEP7 column (tyrosinase); P8, purified through αPEP8 column (TRP2); P13, purified through αPEP13 column (silver protein).

Table I
Melanogenic activities of immuno-affinity purified proteins

| Protein       | Tyrosine oxidase | DOPA oxidase | DOPAchrome oxidase | DHICA oxidase | Melanin formation |
|---------------|------------------|--------------|--------------------|---------------|-------------------|
| TRP1          | 8 ± 5 (n = 5)    | 28 ± 23 (n = 4) | 0 ± 0 (n = 3)    | 131 ± 269 (n = 4) | 239 ± 128 (n = 4) | 0.3 ± 0.2 (n = 5) |
| Tyrosinase    | 325 ± 57 (n = 5) | 3542 ± 809 (n = 4) | 30 ± 4 (n = 3) | 863 ± 209 (n = 4) | 25 ± 14 (n = 4) | 80.7 ± 25.8 (n = 5) |
| TRP2          | 20 ± 13 (n = 5)  | 136 ± 72 (n = 4) | 243 ± 115 (n = 3) | 40 ± 149 (n = 4) | 232 ± 129 (n = 4) | 1.7 ± 1.0 (n = 5) |
| Silver protein| 6 ± 5 (n = 5)    | -6 ± 25 (n = 4) | 0 ± 0 (n = 3)    | 67 ± 134 (n = 4) | 4 ± 5 (n = 4) | -0.3 ± 0.4 (n = 5) |

Fig. 5. Effect of glycosylation inhibitors on the processing of melanogenic proteins. Melan-a cells were incubated with 1 μg/ml swainsonine (S) or 200 μg/ml deoxymannojirimycin (D) for 1 h and then labeled with [35S]methionine for 30 min and chased for 0 or 1 h in the presence of those drugs. Labeled cells were solubilized and analyzed by immunoprecipitation as detailed for Fig. 2A.

αMX, the silver protein seemed not to be lost completely, but to be delivered and localized in melanosomes as an internal, matrix protein (19). We therefore isolated subcellular fractions of melanocytic cells and examined the localization of the silver protein therein. In fact, the silver protein was recognized by αPEP13 in the fractions of isolated melanosomes as were the other TRPs (Fig. 9). Considering the rapid loss of the αPEP13 epitope in vivo (shown above), the newly synthesized silver protein must be delivered to melanosomes very quickly, probably within a few hours. Tyrosinase, TRP1, and TRP2 have been proposed to be transferred to melanosomes via coated vesicles and were highly represented in the purified coated vesicle fraction, but little silver protein was detected by αPEP13 in that fraction (Fig. 9). Studies in progress using immunoelectron microscopy support the concept that the silver protein is localized to spherical vacuoles and is also present within the internal structure of stage II or III melanosomes, but is not detectable in fully melanized stage IV melanosomes (data not shown).

Discussion

We have generated an antibody (αPEP13) against the predicted carboxyl terminus of the protein encoded by the silver locus which recognizes that protein specifically. Our data on the expression of the silver protein using αPEP13 demonstrate that the putative protein is in fact expressed and that its expression is restricted to cells of melanocytic origin. Our studies show further that the silver protein has no known melanogenic...
activities and that while it is an internal component of melanosomal matrix, it is delivered to melanosomes in a manner distinct from the tyrosinase-related proteins. Due to recent advancements in molecular biology and biochemistry, many genes which affect murine coat color have now been cloned and characterized. Some of those genes specifically regulate melanogenesis in melanocytes; albino, brown, slaty, dilute, pink-eyed dilution, silver, and so on. The proteins encoded by the albino, brown, and slaty loci (tyrosinase, TRP1, and TRP2, respectively) have distinct enzymatic functions. Tyrosinase, the activity most critical to melanogenesis, has tyrosine hydroxylase, DOPA oxidase and DHI oxidase activities (27, 36). The DOPAchrome tautomerase activity of TRP2 (17) and the DHICA oxidase activity of TRP1 (18) are known to modulate the catalytic function at a more distal point in the melanogenic pathway than TRP1; such an activity may be required for the polymerization of DHICA metabolites to melanin (37, 38). On the basis of these observations, and the distinctive pattern of its subcellular localization, the silver protein seems likely to be an integral component of melanosomes, with a function distinct from that of the TRP family. These studies further show that the silver protein is synthesized, and transported quickly to the melanosomal fraction. It is subsequently quickly processed to lose its carboxyl terminus (and thus its recognition by aPEP13) although it is not completely degraded and remains within the melanosome. Since the reactivity with aPEP13 is lost relatively quickly due to rapid processing of the silver protein wherein the carboxyl terminus is cleaved, we are unable at this time to determine whether the processed silver protein remains an integral part of the melanizing melanosome or is lost to the cytoplasm and/or to the extracellular milieu. In light of the rapidly emerging literature on the importance of the silver protein (termed gp100 in humans), as well as the tyrosinase related proteins, as specific targets for humoral and cellular immune responses to melanoma, such information takes on an added significance (39, 40). Further studies of the silver protein using the various antibodies now available should provide valuable new information about the formation of premelanosomal structural proteins, the origin of stage I melanosomes and the intracellular trafficking of TRPs to melanosomes.

It is known that the silver mutation has a genetic interaction with the brown locus (1, 41). The brown protein (i.e. TRP1) is multifunctional and may play a structural role in addition to its catalytic role. Since some mutations at the brown and silver loci have similar phenotypes, both proteins might act in series in

![Fig. 6. Degradation of melanogenic proteins in melanocytes (left) or cell lysates (right). (Left) melan-a cells were metabolically labeled by [35S]methionine for 30 min and chased in the complete medium for the time period noted. The same number of trichloroacetic acid-precipitable counts of the labeled cell extracts were subjected to immunoprecipitation with antibodies in each lane, and specifically radiolabeled proteins were analyzed by gel electrophoresis and immunoprecipitation with antibodies in each lane, and specifically radiolabeled counts of the labeled cell extracts were subjected to nitrocellulose membranes, and examined by Western immunoblotting as described under "Experimental Procedures."](image)

![Fig. 7. Cell specific immunoreactivity of αMX. Immunoreactivity of αMX in four cell types was analyzed by metabolic labeling and immunoprecipitation (A) and by Western immunoblotting (B) as detailed for Fig. 2. F, NIH 3T3 fibroblasts; S, Meth A sarcoma cells; B, B16 F10 melanoma cells, M, melan-a melanocytes (wild type); melan-si, melan-si melanocytes (silver mutant).](image)
the melanogenic pathway so that a mutation in either gene affects the same type of melanin production. In melanosomes, TRP1 might interact with the silver protein. Although the brown mutation has no effect on the expression or degradation of the silver protein (data not shown), their products might interact at the protein level, a possibility that will be studied in the future.

We hypothesize that the silver protein functions as a component of the melanosomal matrix, which limits melanogenesis to that intracellular compartment and protects melanocytes from harmful melanogenic intermediates. This in no way obviates a potential novel melanogenic function for the silver protein; on the contrary, as noted above, preliminary studies in other laboratories have suggested that the silver protein may function as a polymerase for DHICA or one of its metabolites (37, 38). Mutations at the silver locus might result in the loss of that function and elicit effects toxic to melanocytes. However, other mechanisms could be proposed to explain such toxic effects. First, the silver protein may have a novel enzymatic function which excludes or decreases the production of harmful melanogenic intermediates (e.g., an anti-oxidant function), although, in this study, we could observe no such melanogenic enzymatic function. Mutations at the silver locus might then lead to the loss of those activities and result in cell death. Because the silver mutation is recessive, however it is unlikely that the mutated silver protein actively produces a toxic substance. Second, it is possible that the mutated silver protein could be an antigen recognized by cytotoxic T-lymphocytes, which might then attack and kill the melanocytes. It has long been thought that disorders such as vitiligo result from the unscheduled destruction of melanocytes by immune mechanisms, and the presence of immune effector mechanisms which target peptides of the silver protein give added credence to this possibility (42, 45). Aberrant trafficking or processing of the mutated silver protein could result in its presentation to the immune system as an antigen, thus prompting a progressive autodestruction of melanocytes.

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34. Orlow, S. J., Zhou, B. K., Reisay, R. E., and Pifko-Hirst, S. (1993) J. Invest. Dermatol. 101, 141–144
35. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106
36. Körner, A. M., and Pawelek, J. (1982) Science 217, 1163–1165
37. Chakraborty, A. K., Kwon, B. S., Bennett, D. C., and Pawelek, J. M. (1994) Pigm. Cell Res. 7, S15
38. Hou, L., Pickard, R. T., and Kwon, B. S. (1994) Pigm. Cell Res. 7, S15
39. Adema, G. J., de Boer, A. J., Vogel, A. M., Loenen, W. A. M., and Figdor, C. G. (1994) J. Biol. Chem. 269, 20126–20133
40. Houghton, A. N. (1994) J. Exp. Med. 180, 1–4
41. Bennett, D. C. (1993) Int. Rev. Cytol. 146, 191–260
42. Bakker, A. B. H., Schreurs, M. W. J., de Boer, A. J., Kawakami, Y., Rosenberg, S. A., Adema, G. J., and Figdor, C. G. (1994) J. Exp. Med. 179, 1005–1009
43. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T., and Rosenberg, S. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6458–6462