Mouse *silver* mutation is caused by a single base insertion in the putative cytoplasmic domain of Pmel 17

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ABSTRACT

This laboratory has established in previous studies that Pmel 17, a gene expressed specifically in melanocytes, maps near the *silver* coat color locus (*si/si*) on mouse chromosome 10. In the current study, we have focused on determining whether or not the *si* allele carries a mutation in Pmel 17. Pmel 17 cDNA clones, isolated from wild-type and *si/si* murine melanocyte cDNA libraries, were sequenced and compared. A single nucleotide (A) insertion was found in the putative cytoplasmic tail of the *si/si* Pmel 17 cDNA clone. This insertion is predicted to alter the last 24 amino acids at the C-terminus. Also predicted is the extension of the Pmel 17 protein by 12 residues because a new termination signal created downstream from the wild-type reading frame. The mutation was confirmed by the sequence of the PCR-amplified genomic region flanking and including the mutation site. The fact that *si/si* Pmel 17 was not recognized by antibodies directed toward the C-terminal 15 amino acids of wild-type Pmel 17, indicated a defect in this region. We conclude from these results that *silver* Pmel 17 protein has a major defect at the carboxyl terminus. The chromosomal location and the identification of a potentially pathologic mutation in *si*-Pmel 17 support our conclusion that Pmel 17 is encoded at the *silver* locus.

INTRODUCTION

The Pmel 17 gene, located on mouse chromosome 10 near the *si* (*silver*) locus (1), is preferentially expressed in melanocytes (2). Pmel 17 was initially cloned from a human melanocyte cDNA library by screening with polyclonal antibodies against tyrosinase, a key enzyme in melanin synthesis encoded by the *c* locus on mouse chromosome 7 (3). The Pmel 17 sequence contains a

hydrophobic domain at the C-terminus suggesting a membrane-localization, possibly in melanosomes, the site of melanin production. The differential expression in melanocytes, and the enhanced expression of Pmel 17 with melanogenic stimulation (2) suggest that Pmel 17 belongs to the family of melanogenic enzymes that includes tyrosinase, the *b*-locus protein/gp75, and DOPAchrome tautomerase (3–5). As a first step in assigning a melanogenic function to Pmel 17, we examined whether DNA from *si/si* mouse, which are diluted in coat color, contains a mutation in Pmel 17 that might lead to dysfunction of the protein.

Toward this aim we constructed cDNA libraries from melanocyte mRNA of wild-type and *si/si* melanocytes and isolated Pmel 17 cDNA clones. Sequence analysis demonstrated a single base (A) insertion at the 3'-terminus in *silver* Pmel 17 cDNA, with a predicted change in 24 amino acids and extension of the protein by 12 residues. Since antibodies against the C-terminal tail of Pmel 17 did not recognize the mutant protein, we were able to confirm that *silver*-Pmel 17 has a major defect in this domain.

MATERIALS AND METHODS

Mouse melanocyte culture

Melanocytes were isolated from the skin of newborn *si/si*, wild-type C57BL/6J and B10.BR, and from *Himalayan* mice carrying mutation at the albino *c* locus (*c^h/c^h*). These were grown in Ham's F-10 medium (American Biorganics, North Tonawanda, NY), supplemented with penicillin (200 U/ml), streptomycin (100 µg/ml), L-glutamine (1 mM), horse serum (10%) and newborn calf serum (2.5%) (both from GIBCO), phorbol 12-myristate 13-acetate (85 nM, GIBCO) and dbcAMP (0.1 Mm, Sigma) (6). These growth conditions allow the expression of wild-type as well as mutant melanocyte melanogenic functions.

Southern blot hybridization

High molecular weight murine genomic DNA was prepared from splenocytes as described (3), digested with restriction endonu-

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cleases, electrophoresed in 1% agarose gels at 4°C, transferred to Gene Screen Plus membrane (DuPont/NEN) (7), and hybridized to a ³²P-labeled wild-type mouse Pmel 17 cDNA probe at 65°C. The blot was then washed twice with 2 × standard saline citrate (SSC) at room temperature for 5 min, twice with 2 × SSC/1% SDS at 65°C for 30 min, and twice with 0.1 × SSC at room temperature for 30 min, and autoradiographed for 2 days at -80°C.

For Northern blots, poly(A)⁺ selected RNAs from mouse melanocytes were fractionated in a formaldehyde/1.4% agarose denaturing gel (8) and transferred to Gene Screen Plus membrane. The blot was hybridized to [³²P]ATP-labeled wild-type mouse Pmel 17 cDNA in a mixture of 50% (vol/vol) formamide/10% (wt/vol) dextran sulfate/1 M sodium chloride/1% (wt/vol) SDS/100 µg of sheared salmon sperm DNA.

Construction and screening of cDNA libraries

cDNAs were constructed from poly(A)⁺ selected RNAs prepared from *silver* and wild-type melanocytes and cloned in λgt11 vector (9,10). The cDNA libraries were screened with a [³²P]ATP-labeled human Pmel 17 cDNA probe. Positive cDNA clones were isolated and further characterized by restriction analysis and DNA sequencing.

DNA sequencing

Mouse Pmel 17 cDNAs and PCR-fragments of mouse wild-type and *si/si* genomes were subcloned in the PGEM 7 vector (Promega). Each subclone was sequenced after generating unidirectional deletions using the Erase-a-Base system (Promega). The nucleotide sequence was determined by the dideoxy chain termination method (11) using Sequenase Version 2.0 (US Biochemicals, Cleveland, OH).

Genomic PCR

The Pmel 17 genomic segments defined by a set of forward (5'-CTGCTTCTACACCAAGTG-3') and reverse (5'-GTCAACCCCAGGAAAT-3') primers were amplified by PCR using C57BL/6J and *silver* mouse genomic DNA. The 18-base forward primer represents codons from 519 to 524 and the 16-base reverse primer is taken from 3' untranslated region of the Pmel 17 cDNA sequence. The genomic segments amplified from C57BL/6J and *silver* DNA were sequenced.

Antibodies, immunoprecipitation and immunoblotting

Anti-Pmel 17N serum was obtained from rabbits immunized with a recombinant protein representing amino acids 141 to 443 of mouse Pmel 17, encompassing the repetition motif (12). Anti-Pmel 17C serum was raised by immunizing rabbits with a synthetic peptide spanning the carboxy-terminal 15 amino acids (13). Immunoprecipitation and immunoblotting procedures were carried out using standard protocols as described (14).

RESULTS

Restriction fragment length polymorphism

Southern blot analysis was performed to determine whether the *silver* mutation was associated with RFLP in the Pmel 17 locus, using DNA purified from homozygous normal (+/+), *silver*

mutant (*si/si*), or heterozygous *si/+* littermate. A polymorphism was detected after digestion with *Eco*RI and *Hind*III restriction enzymes. In normal littermate (+/+) DNA, the Pmel 17 probe hybridized to 2.0-kb *Eco*RI and 2.8-kb *Hind*III fragments. In contrast, the digested *silver* homozygote (*si/si*) DNA showed 1.8-kb *Eco*RI and 3.0-kb *Hind*III fragments, while heterozygote (*si/+*) DNA exhibited 2.0-kb/1.8-kb *Eco*RI and 2.8-kb/3.0-kb *Hind*III fragments (data not shown). The restriction enzyme *Pvu*II did not produce any polymorphism. These results show that the 1.8-kb *Eco*RI and 3.0-kb *Hind*III bands co-segregate with the *silver* mutation and indicate that a large deletion or rearrangement is not present in the mutant allele. This conclusion is supported by Northern blot analysis showing that *silver* and wild-type Pmel 17 RNA are similar in size and abundance (data not shown).

Sequence analysis of wild-type Pmel 17 cDNA

The deduced amino acid sequence of wild-type Pmel 17 are shown in Figure 1A. The open reading frame encodes a protein of 626 amino acids, shorter than its human homologue by 42 residues. Since the first 23 amino acids have the characteristics of a signal peptide (Fig. 1B), fitting the -1, -3 rule (15) to be cleaved, the final protein is predicted to consist of 603 amino acids with a protein backbone *M_r* of ~64,000. A stretch of 28

A

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1  MUGVORRSEF PVLVL SALLA VGALGSRNQ DWLGVPRLV TKTWNRQLYP
51  EWTEVQGSNC WRGGQVSLRV INDGPTLVGA NASFSIALHF PGSQKVLPGD
101 QVIWANTTII NSSQVWGGQP VYPQEPDDAC VFPDGGPCPS GPKPKRFSFV
151 YVWKTWGYW QVLGGPVSRS SIATRHAKLG THTEMTVYH RRGQSQYVPL
201 AHASSTFTIT DQVPFSVSVS QLQALDGETK HFLRNHPLIF ALQLHDPSGY
251 LAEADLSYTW DFGDGTGTLI SRALDVTHTY LESGSVTAQV VLQAAIPLVS
301 CGSSPVP GTT DGYMPTAEAP GTTSROGTTT KVVGTTTGGOM PTTOPSGTTV
351 VOMPTTEVTA TTSEOML TSA VIDTTLAEVS TTEGTGTTPT RPSGTTVAQA
401 TTTEGPDASP LLPTQSSTGS ISPLDDTDT IMLVKRQVPL DCVLYRYGSF
451 SLALDIVQGI ESAEILQAVP FSEGDAFELT VSCQGGLPKE ACMDISSPGC
501 QPPAQRCLQS VPPSPDCQLV LHQVLKGGSG TYCLNVS LAD ANSLAVASTQ
551 LVVPGQDGLL GOAPLLVGIL LVLVAUVLAS LILGIDLRSR AQFPKCHMVA
601 LTAAPASGLR ARGLENSPL LSGQQV

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Figure 1. Amino acid sequence analysis of the potential Pmel 17 protein. (A) Deduced amino acid sequence of murine Pmel 17. The signal peptide region is underlined with a heavy line. The potential glycosylation sites are indicated with shadow. The putative transmembrane region is doubly underlined. Two repeats of 26 amino acids are underlined. (B) Hydropathicity profile of the deduced amino acid sequence of murine Pmel 17. Local hydropathicity values calculated by the method of Kyte and Doolittle were plotted versus amino acid residues. Positive values indicate hydrophobic regions, and negative values indicate hydrophilic regions. Amino acid positions are indicated by numbers below the figure. Signal peptide region, two repeat motifs, and transmembrane regions are indicated by bars with numbers 1, 2 and 3, respectively. The murine Pmel 17 sequence has been deposited in the Gene Bank database (Accession No. U14133).

Figure 2. Schematic representation of the potential Pmel 17 protein amino acid changes in the *silver* Pmel protein. The entire coding region is boxed. The potential signal peptide is hatched. The transmembrane segment is indicated by horizontal lines. The central repeat motifs are stippled. The possible N-linked glycosylation sites (CHO) are indicated. The positions of eight potentially harmless point mutations seen in *silver* Pmel cDNA are shown by (+). The nucleotide substitutions, codons involved and the consequent amino acids are indicated below the figure. A vertical arrow and a star (*) shows the position of the A insertion in the *silver* Pmel 17 sequence. The difference in amino acid sequences between C57BL/6 and *silver* Pmel are indicated below the figure.

amino acids constituting a hydrophobic domain is present toward the C-terminus (amino acids 558–585) (Fig. 1A and 1B). This region is flanked by charged residues at each end (Asp 557 and Asp 559), a feature consistent with a transmembrane domain. There are four potential N-linked glycosylation sites at amino acid positions 81, 106, 111 and 535. Alignment of human and mouse Pmel 17 amino acid sequences showed 77% identity, with an overall high similarity in structure including a repeat motif in the middle of the protein. Whereas human Pmel 17 contains 3 repeats, the mouse counterpart contains only two (Fig. 1A). The 26-amino acid repeats show a hydrophobicity pattern similar to one another (Fig. 1B). The highest diversity in amino acid sequence between human and mouse proteins lies in the repeat motif, suggesting that this domain may not participate in an enzymatic function.

Identification of the *si/si*-Pmel 17 mutation

The *silver*-Pmel sequence differs from wild-type Pmel 17 by nine nucleotide substitutions and one insertion. The substitutions were detected at nucleotide positions 294, 508, 509, 523, 1117, 1188, 1303, 1412 and 1593 with four predicted amino acid changes. These changes were Ser → Leu (codon 170), Arg → Gly (codon 175), Asp → Asn (codon 373) and Phe → Ser (codon 471) (Fig. 2). When we examined the amino acid sequences of human Pmel 17 at these positions, codons 170, 175 and 471 were Leu, Gly and Ser, respectively. The change at codon 373 is conservative. Therefore, these changes may not be harmful for the function of Pmel 17, and may be regarded as polymorphisms. However, an insertion at the putative cytoplasmic tail, where an A residue was found between nucleotides 1806 and 1807 after codon 602, is predicted to shift the reading frame at the C-terminus, altering the last 24 amino acids and creating a new termination codon that extends the protein by 12 amino acids. (Fig. 2)

To confirm the insertional mutation in the *si* genomic sequence, we amplified the genomic region that contained the mutation site by polymerase chain reaction (PCR). Two synthetic oligonucleotides were used as specific primers to amplify ~1.3-kb segments of wild-type and *silver* DNAs. Sequencing of these fragments revealed two introns in this region and confirmed the A insertion in the genomic DNA of *silver* (Fig. 3).

Immunological analysis of Pmel 17 proteins

The predicted sequence alteration due to the A insertion was corroborated by immunological analysis. Antibodies that recognized the carboxyl terminus of wild-type Pmel 17 did not react with the protein encoded by the *si* allele (Fig. 4). In contrast, the antibodies against the N-terminal region recognized both C57BL/6J and *silver* Pmel 17. These results support the conclusion that *silver* Pmel 17 has a major defect at the carboxyl terminus.

DISCUSSION

We are reporting data which show that a mutation in Pmel 17 causes the *silver* phenotype. Although the mechanism of silvering is not yet clear, analysis of *si/si* mouse skin suggests an effect on melanocyte viability, because of a lower number of melanocytes (16). This reduction in melanocyte density may be caused by the premature death of pigment cells during the hair cycle (17). It is possible that the *silver* mutation affects the ability of Pmel 17 to interact with other proteins, especially with *b*-locus protein/gp75, because the effect of the *silver* mutation was greatest on a *B/b* background (18). An analysis of the effects of mutations in the catalytic domain of Pmel 17 or the behavior of melanocytes in Pmel 17 knockout mice may define a more precise role for this protein.

Figure 3. Portion of sequence gel containing the A-insertional mutation. The mutation site is indicated by an arrow and a bold letter. The sequence is labeled 5' and 3' in reference to the orientation of the Pmel 17 gene. Although the sequence gel shown is difficult to determine whether the correct sequence is **AGCT**- or **ACGT**- starting at the insert A, sequencing the other strand shows that the sequence given in this figure is correct.

Figure 4. Immunological analyses of wild-type and *silver*-Pmel 17 proteins. Rabbit polyclonal antibodies against the carboxyl terminal 15 amino acids of normal mouse Pmel 17 protein ($\alpha 17C$) or preimmune serum (PI) were used in the immunoprecipitations (ip) and polyclonal antibodies against the N-terminus repetition motif area ($\alpha 17N$) were used for immunoblotting (ib). For immunoprecipitation, B10.BR wild-type (WT) and *silver* (*si/si*) melanocytes were metabolically labeled with Tran- ^{35}S -label (ICN Biomedicals, Inc., Costa Mesa, CA, 1132 Ci/mmol) (500 μ Ci/ml) for 3 h and approximately 1×10^7 c.p.m. in TCA-precipitable material were applied per immunoprecipitation. Immune complexes were separated by electrophoresis in 8% polyacrylamide gel, followed by fluorography. For immunoblotting, *silver* and wild-type melanocytes were lysed in PBS containing 1% NP-40, 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 12,000g for 5 min. The supernatant containing approximately 100 μ g of protein was denatured by boiling for 2 min in 50 μ l SDS-sample buffer and the proteins were resolved on 8% polyacrylamide gel. Prestained molecular weight markers (BioRad) are shown in kDa (K) on the left. Arrow heads indicate the mouse Pmel 17 band. Because the anti-Pmel 17 antibodies are polyclonal rabbit serum, background bands common to all the lanes appear above and below the Pmel 17 band.

The single-base A insertion in the *silver* Pmel 17 allele predicts a change in the amino acid sequence at the 3'-half of the cytoplasmic domain. This prediction is supported by the immunological studies. The anti-Pmel 17c serum which recognized the

carboxyl terminus of the murine Pmel 17 was prepared by immunizing the synthetic peptide predicted from the cDNA analysis of this study. Further studies are in progress to show the size difference between the wild and *silver* Pmel 17 proteins and to generate an antibody that is specific for the predicted C-terminal extension of *silver* Pmel 17. Nevertheless, the anti-Pmel 17c antibody, later called anti-PEP 13 (13) was used to show that *silver* locus product is a melanosomal matrix protein and that anti-PEP 13 did not detect *silver* locus product by others (13,19).

Pmel 17 protein has been suggested to have an enzymatic activity which promoted the conversion of 5,6-dihydroxyindole-2-carboxylic acid to melanin (20,21). If Pmel 17 is an enzyme involved in melanin biosynthesis, the catalytic function will be in the protein region between N-terminus and transmembrane domain which resides within the melanosome, and *silver* Pmel 17 will be expected to retain full enzymatic activity. The full melanogenic phenotype of cultured *silver* melanocytes supports this notion with its apparent impairment in growth rate (18). The findings that Pmel 17 mRNA expression is induced by IBMX and β MSH and the mRNA levels correlate with melanin content (2), suggest that Pmel 17 is regulated by the same environmental stimuli that control *tyrosinase*.

Recently, we proposed that at least two families of genes regulate the melanin biosynthesis (12). The first is the tyrosinase gene family which is composed of tyrosinase, *b*-locus/gp75 and DOPAchrome tautomerase. The second regulator is the Pmel 17 gene family. Human Pmel 17 shares several similarities with chicken MMP 115, including the repeat motif in the middle of the proteins (12,22). Nevertheless, MMP 115 is not a straight-forward chicken homologue of Pmel 17, because it does not contain a putative membrane-spanning domain. While MMP 115 is a melanosomal matrix protein in the chicken (23), human Pmel 17 has been localized to the melanosomal membrane and the melanosomal matrix (Dr Gisela Moellmann, personal communication).

The human Pmel 17 protein is apparently processed in the proteosomes of normal and malignant melanocytes and its peptides are presented by HLA-A2 antigen. These peptides become tumor antigens which is recognized by cytotoxic T cells of certain melanoma patients (24,25). It remains to be determined whether a certain haplotype of murine Class I MHC antigen can present the Pmel 17-derived peptides in the melanocytes.

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