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Genetics

Identification of a melanosomal membrane protein encoded by the pink-eyed dilution (type II oculocutaneous albinism) gene

(coat color/melanocyte/tyrosinase/tyrosine transport)

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ABSTRACT **The pink-eyed dilution (*p*) locus in the mouse is critical to melanogenesis; mutations in the homologous locus in humans, *P*, are a cause of type II oculocutaneous albinism. Although a cDNA encoded by the *p* gene has recently been identified, nothing is known about the protein product of this gene. To characterize the protein encoded by the *p* gene, we performed immunoblot analysis of extracts of melanocytes cultured from wild-type mice with an antiserum from rabbits immunized with a peptide corresponding to amino acids 285–298 of the predicted protein product of the murine *p* gene. This antiserum recognized a 110-kDa protein. The protein was absent from extracts of melanocytes cultured from mice with two mutations (p^{cp} and *p*) in which transcripts of the *p* gene are absent or greatly reduced. Introduction of the cDNA for the *p* gene into p^{cp} melanocytes by electroporation resulted in expression of the 3.3-kb mRNA and the 110-kDa protein. Upon subcellular fractionation of cultured melanocytes, the 110-kDa protein was found to be present in melanosomes but absent from the vesicular fraction; phase separation performed with the nonionic detergent Triton X-114 confirmed the predicted hydrophobic nature of the protein. These results demonstrate that the *p* gene encodes a 110-kDa integral melanosomal membrane protein and establish a framework by which mutations at this locus, which diminish pigmentation, can be analyzed at the cellular and biochemical levels.**

Melanin biopolymers determine eye, hair, and skin color in mammals and are synthesized by specialized cells termed melanocytes. The pink-eyed dilution (*p*) locus has long been known to control the quantity of melanin made by melanocytes and to influence the shape and size of the melanosome (1–3), the organelle in which melanin synthesis and deposition occurs.

Recently, the murine *p* gene and its human homologue were cloned (4, 5). Mutations in the human *P* gene are responsible for type II (tyrosinase-positive) albinism, the most common form of oculocutaneous albinism (6, 7). In addition, genetic and molecular evidence supports the contention that the hypopigmentation observed in both the Angelman and Prader–Willi syndromes may be due to molecular deletions that include the *P* locus (4–7). Despite these recent molecular advances, the protein product of the *p* gene has not yet been identified.

The predicted product of the *p* gene is a unique protein with 12 transmembrane domains (4, 5). Many proteins previously described with such a secondary structure function as transmembrane channels or transporters. It has been suggested that the *p* protein may function as a transporter of tyrosine because (*i*) culture in high concentrations of tyrosine induces pigmentation in ocular melanocytes from mice homozygous for the *p* allele, a null-phenotype *p* mutation (8), and (*ii*) incubation of hair follicles from persons with type II oculocutaneous albinism in high concentrations of tyrosine or dopa results in melanin deposition (9). Although homology to a bacterial tyrosine transporter has been suggested (5), we were unable to confirm this homology by rigorous analysis employing the Monte Carlo method (10).

However, not all the effects of mutations at the *p* locus are readily explained by a transport function. Diminished levels of tyrosinase activity in extracts of the skins of mice carrying the *p* mutation, even in the presence of exogenous tyrosine, have long been recognized (11, 12). We have recently shown that the postnatal expression of tyrosinase and the tyrosinase-related proteins TRP-1 and TRP-2 is markedly diminished in mice homozygous for the p^{un} mutation (pink-eyed unstable) (13), in which a duplication disrupts the linear order of exon sequences and, hence, transcription of the *p* gene (4, 14, 15). In addition, the high molecular weight forms of the TRP family of melanosomal membrane proteins present in wild-type mice (16) are not detectable in the eyes of p^{un}/p^{un} mice (13). Thus, the p protein may play other important roles in the melanosome.

We have employed an anti-peptide antiserum in combination with melanocytes cultured from genetically defined mice to demonstrate that the *p* gene encodes a 110-kDa integral melanosomal membrane protein. This protein is absent from melanocytes cultured from two strains of mutant mice in which the *p* gene transcript is not expressed. Our results form a framework that will allow us to address the role of this protein in melanogenesis and to analyze the effects of mutations that cause type II albinism in humans at the biochemical and cellular levels.

METHODS

Immunologic Reagents and Techniques. The peptide NH_2-RTFEIVSREAVSIS-CO_2H, corresponding to aa 285–298, which are contained within the first intraluminal loop of the predicted protein product of the murine *p* locus, and the peptide NH_2-KDIRLASAVLEVEL-CO_2H, corresponding to aa 6–19, which are contained within the predicted first cytoplasmic domain (ref. 4 and GenBank accession no. M97900), were synthesized by the Harvard Microchemical facility (Cambridge, MA). Rabbits were immunized with peptides crosslinked to cationized (Pierce) bovine serum albumin and to keyhole limpet hemocyanin by using the Imject SuperCarrier system according to the manufacturer's

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Abbreviations: TRP, tyrosinase-related protein; LGF, large granule fraction.

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instructions (Pierce). Rabbits were also immunized with a protein A fusion conjugate expressed in *Escherichia coli* by using the pEZZ expression vector (Pharmacia LKB). The fusion protein was purified on IgG-Sepharose and corresponded to aa 1–170 of the predicted murine *p* gene product (ref. 4 and GenBank accession no. M97900). Anti-peptide antisera αPEP7 (C terminus of tyrosinase), αPEP1 (C terminus of TRP-1), and αPEP8 (C terminus of TRP-2) were the gift of V. Hearing (National Cancer Institute, Bethesda). Protein (20 μg per lane) was subjected to SDS/PAGE on 7.5% gels and immunoblot analysis was performed as described (17).

Cell Culture. Melanocytes cultured from C57BL/6 mice (melan-a cells) and from albino mice (melan-c cells) were obtained from D. Bennett (St. George's Hospital, London). Melanocytes from C57BL/6J (wild-type) and from 129/J mice (genotype *pp* cc^{ch}) were provided by R. Halaban (Yale University Skin Disease Research Center, New Haven, CT).

Melanocytes were cultured from the skin of newborn mice homozygous for the p^{cp} (pink-eyed cleft-palate) mutation by a modification of the method of Halaban *et al.* (18). One- to 3-day-old mice were killed, washed in 70% EtOH, and then rinsed in sterile PBS without Ca^{2+} and Mg^{2+}. The dorsal skin was removed, minced in Eagle's minimal essential medium supplemented with 0.25% trypsin (GIBCO), and incubated at 37°C; after 2 h, the epidermis was peeled off and discarded. The medium and dermis were transferred to a 15-ml conical tube and pipetted repeatedly until the medium was cloudy, and debris was allowed to settle. The supernatant was placed in another 15-ml conical tube and centrifuged at low speed on a table-top centrifuge (700 × *g*, 10 min). The supernatant was aspirated, and the pellet was resuspended in 3 ml of melanocyte medium [Ham's F-10 medium containing 20% (vol/vol) fetal calf serum (Atlanta Biologicals, Norcross, GA), 48 nM phorbol 12-myristate 13-acetate, 0.1 mM isobutylmethylxanthine, 0.2 μM β-melanocyte-stimulating hormone, 0.1 mM dibutyryl-cAMP (Sigma), penicillin (50 units/ml), kanamycin (100 μg/ml; GIBCO), and streptomycin (50 μg/ml; Sigma)] and transferred to a 25-cm^2 flask. The debris was suspended in 3 ml of melanocyte medium, pipetted, allowed to settle, and transferred to the same 25-cm^2 flask. The cells were incubated at 37°C, and after 24–48 h, the medium was aspirated, and fresh melanocyte medium was added. Contaminating fibroblasts were removed by incubating the culture for 2 or 3 days in melanocyte medium supplemented with Geneticin (G418 sulfate, Sigma; 80 μg/ml). The cells were fed twice a week and were split 1:3 every 10–14 days.

Northern Blot Analysis. Total cellular RNA was extracted by the RNAZol B method as recommended by the supplier (Cinna/Biotecx Laboratories, Friendswood, TX). The RNA (1–10 μg) was separated by electrophoresis on a 1% agarose/16% (vol/vol) formaldehyde gel and transferred to a nylon membrane (Hybond-N+, Amersham). A probe consisting of the 2.1-kb *Sst* I fragment from the *p* gene cDNA clone MC2701 (4) was labeled to a specific activity of 3×10^9 cpm/μg by using a Prime-It II kit (Stratagene), and the probe [5×10^6 cpm/ml in phosphate-buffered 7% (wt/vol) SDS hybridization solution (19)] was hybridized to the membrane. The blot was washed with 0.2× SSC/0.1% SDS at 62°C, prior to exposure to x-ray film.

Cell Transfection. The mouse *p* cDNA insert from clone MC2701 (4) was subcloned into the *Bam*HI site of the expression vector LXSN (20), which allows high constitutive expression of the cDNA under the control of Rous sarcoma virus long terminal repeat early region promoter sequences. Approximately 1×10^7 melanocytes from p^{cp} mice were harvested using 0.25% trypsin (GIBCO). Harvested cells were washed once in Ca^{2+}, Mg^{2+}-free PBS and resuspended in 1 ml of Dulbecco's modified Eagle's medium (GIBCO). LXSN-MC2701 DNA (6 μg) was added to the cell suspension and introduced into cells with a BRL electroporator system 1 at 575 V/cm for 13.2 ms. The cells were transferred to 9 ml of melanocyte medium and then placed into 20-cm^2 plates. The transfected cultures were grown to confluency (2 days) and then split 1:3. Cells were fed every 3 days with melanocyte medium containing Geneticin (0.8 mg/ml), and colonies appearing on day 14 were isolated by using cloning rings and then expanded.

Subcellular Fractionation. Cultured melanocytes were resuspended in 0.25 M sucrose/aprotinin (20 μg/ml)/5 mM benzamidine and homogenized on ice with a glass–glass homogenizer. A postnuclear supernatant was prepared by centrifugation of extracts at 700 × *g* for 10 min. The large granule fraction (LGF) was isolated by centrifugation at 10,000 × *g* for 10 min. The LGF was solubilized by the addition of lysis buffer containing 1% Triton X-114, and insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Supernatants were warmed to 37°C for 5 min and aqueous and detergent phases were isolated by centrifugation at room temperature at 700 × *g* for 5 min (21).

Percoll gradient centrifugation of the postnuclear supernatants of cultured cells was as described (17).

RESULTS

Identification of the *p* Locus Gene Product. An antiserum (hereafter termed α110kDa) raised in rabbits immunized with a peptide corresponding to aa 285–298 of the predicted murine *p* locus gene product recognized a protein of 110 kDa in extracts of cultured melanocytes from black (melan-a) (Fig. 1*A*) and albino (melan-c) (Fig. 1*B*) mice, both genotypically wild type at the *p* locus. The choice of peptides was based upon the prediction that hydrophilic stretches of a protein are more antigenic and more accessible to antibodies. The sequence chosen lacks any amino acids predicted to undergo posttranslational modification such as glycosylation or phosphorylation (4). To determine whether this 110-kDa protein was encoded by the *p* locus, we examined the reactivity of the α110kDa antiserum with extracts of melanocytes from mice homozygous for a deletion of all amino acid encoding sequences of the *p* gene (p^{cp}, pink-eyed cleft-palate) (22) and from 129/J mice homozygous for the *p*

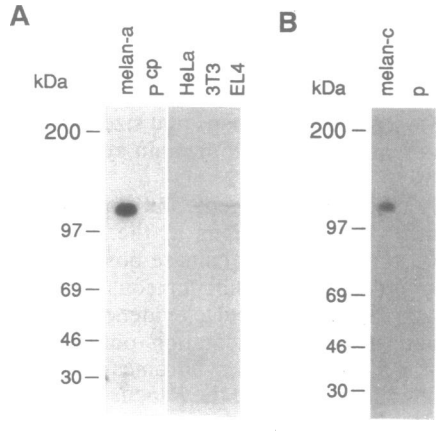

FIG. 1. Immunoblot analysis of *p* locus protein. Equal quantities of protein from postnuclear supernatants from cultured cells were subjected to SDS/PAGE followed by immunoblot analysis with the antiserum raised against the peptide representing aa 285–298 of the predicted *p* locus protein. (*A*) Lanes: melan-a, melanocytes cultured from C57BL/6 mice; p^{cp}, melanocytes cultured from mice homozygous for the p^{cp} mutation; HeLa, human transformed cervical epithelial cells; 3T3, murine fibroblast cells; EL4, human lymphoblastic cell line. (*B*) Lanes: melan-c, melanocytes cultured from albino (c^{2J}/c^{2J}) mice; p, melanocytes cultured from 129/J mice (genotype *ppcc*^{ch}). No reactivity was seen with preimmune rabbit sera.

mutation, which ablates or greatly diminishes transcription of the *p* gene by an as-yet-undetermined mechanism (4, 5). Neither cell line expressed the 110-kDa protein (Fig. 1). The protein was not detected in extracts of cultured cervical epithelial cells, lymphoma cells, or fibroblasts (Fig. 1*A*) or in cultured keratinocytes (data not shown).

In contrast, when a *p* expression vector (LXSN-MC2701) was introduced into p^{cp} melanocytes by electroporation, a 3.3-kb mRNA (the predicted size) and the 110-kDa protein were expressed strongly (Fig. 2).

This anti-peptide antiserum reacted strongly with the reduced denatured 110-kDa protein on immunoblots but did not react with the unreduced or native 110-kDa protein. Therefore, we could not employ the antiserum for immunoprecipitation analysis.

Identification of an Immunologically Related 65-kDa Protein Not Encoded at the *p* Locus. During the course of our studies, we also developed two antisera in rabbits immunized with a protein A fusion bearing the N-terminal 174 aa of the murine p protein. These antisera and a third antiserum (hereafter termed α65kDa) directed against the peptide representing aa 6–19 in the first predicted cytoplasmic domain of the p protein recognized a protein of 65 kDa. We believe that all three antisera recognize the same 65-kDa protein because the abundance and migration of the antigen recognized by each antiserum were always identical to that recognized by the other two (data not shown). In contrast to the 110-kDa protein, the 65-kDa protein was expressed strongly in both p^{cp} (Fig. 2*B*) and *p* (data not shown) melanocytes. Expression of the 65-kDa protein was low in melanocytes cultured from wild-type mice (data not shown). No change in the level of expression of the 65-kDa protein was seen after the *p* locus cDNA was introduced into p^{cp} cells (Fig. 2*B*). Thus, although recognized by antisera raised against a portion of the predicted *p* gene product, this 65-kDa protein must be encoded at an independent genetic locus.

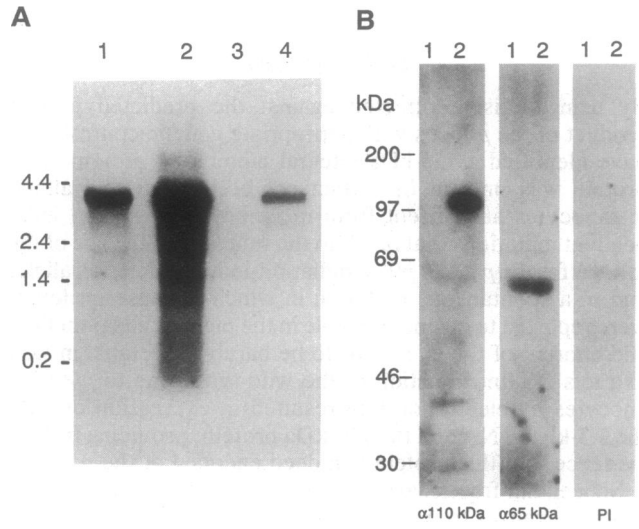

FIG. 2. Introduction of *p* locus cDNA results in expression of 110-kDa protein. Melanocytes from mice homozygous for the p^{cp} mutation and the same cells after introduction of a cDNA encoding the *p* locus were analyzed. (*A*) Northern blot analysis using the 2.1-kb *Sst* I fragment from cDNA clone MC2701. Lanes: 1, 10 μg of total RNA from C57BL/6 wild-type melanocytes (exposure, 12 h); 2, 10 μg of total RNA from C57BL/6 wild-type melanocytes (same as lane 1; exposure, 6 days); 3, 10 μg of total RNA from untreated p^{cp}/p^{cp} melanocytes (exposure, 6 days); 4, 1 μg of total RNA from p^{cp}/p^{cp} melanocytes electroporated with the *p* cDNA construct (exposure, 6 days). The migration of RNA markers (in kb) is shown to the left. (*B*) Immunoblot analysis with α110kDa, α65kDa, and preimmune (PI) sera. Lanes: 1, p^{cp}/p^{cp} cells; 2, same cells after electroporation with the LXSN-MC2701 construct.

Subcellular Localization of the 110-kDa *p* Locus Protein. Subcellular fractionation confirmed the melanosomal distribution of the 110-kDa p protein. It was highly enriched in the LGF, which contains all stages of melanosomes (23) (Fig. 3). In contrast, the 65-kDa protein was exclusively located in the 10,000 × *g* supernatant fraction of melanocytes.

Phase separation of protein solubilized with the nonionic detergent Triton X-114 is a useful technique for separating integral from nonintegral membrane proteins (21). Upon phase separation of the 10,000 × *g* pellet, the 110-kDa protein partitioned into the detergent phase, as expected for a highly hydrophobic integral membrane protein (Fig. 3), as did TRP-1, a *bona fide* melanosomal membrane protein (17). Unlike TRP-1, which was also present in the Triton X-114 detergent phase of the 10,000 × *g* supernatant [presumably in the membranes of small vesicles (24)], the 110-kDa protein was totally absent from the 10,000 × *g* supernatant. In contrast, the 65-kDa protein was entirely limited to the 10,000 × *g* supernatant. Upon Triton X-114 phase separation of this fraction, the protein partitioned into the aqueous phase. Thus, the 65-kDa protein did not behave like an integral membrane protein and its distribution did not overlap that of the 110-kDa protein (Fig. 3).

Further evidence for the localization of the 110-kDa protein to melanosomes was obtained from Percoll gradient centrifugation analysis of the postnuclear supernatants of cultured melanocytes (Fig. 4). When extracts of melan-c (albino) cells, which are totally devoid of melanin, were analyzed, the majority of the 110-kDa protein (Fig. 4*A*) and tyrosinase (Fig. 4*B*) comigrated in a low-density peak near the center of the

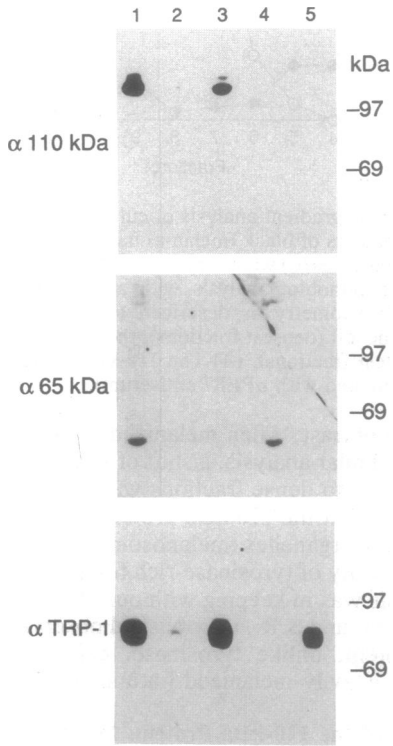

FIG. 3. Subcellular localization of the *p* locus protein. The melan-c cells were subjected to subcellular fractionation, followed by immunoblot analysis with antisera to the 110-kDa protein (α110kDa) encoded by the *p* locus, the 65-kDa protein (α65kDa), and antiserum to TRP-1 (αTRP-1). Lanes: 1, postnuclear supernatant; 2, LGF (melanosome-rich LGF), Triton X-114 aqueous phase; 3, LGF, Triton X-114 detergent phase; 4, 10,000 × *g* supernatant, Triton X-114 aqueous phase; 5, 10,000 × *g* supernatant, Triton X-114 detergent phase. In some experiments including this one and the one in Fig. 5, the 110-kDa protein behaved as a tight doublet rather than a single band.

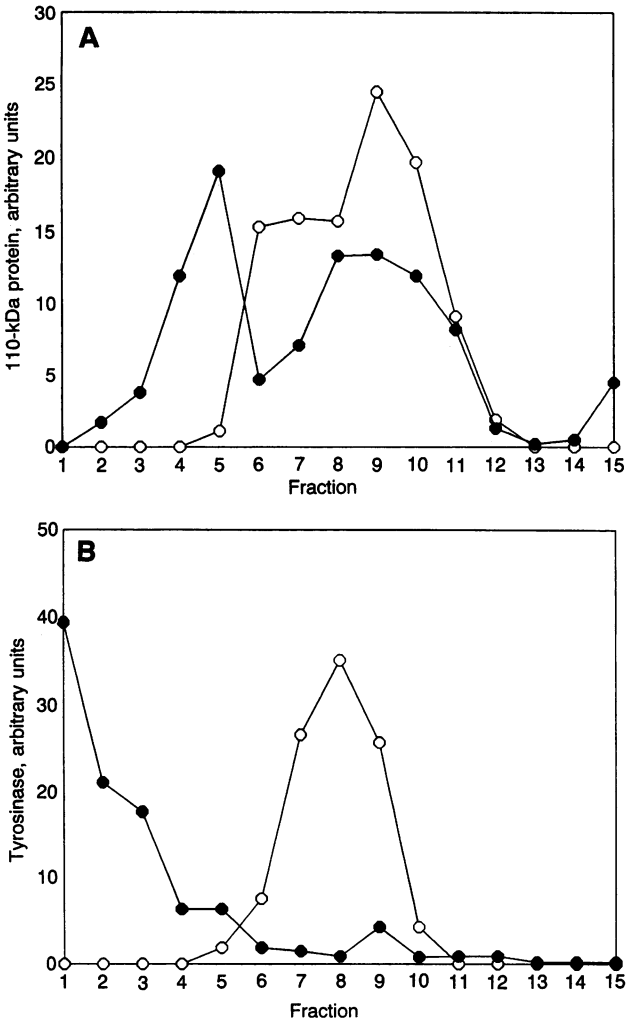

FIG. 4. Percoll gradient analysis of cultured melanocytes. Postnuclear supernatants of black (melan-a) melanocytes (●) and albino (melan-c) melanocytes (○) were subjected to Percoll gradient analysis followed by immunoblot analysis with anti-peptide antisera and quantitative densitometry as described (17). The bottom of the gradient is at the left (densest fractions); the top of the gradient is at the right (lightest fractions). (*A*) The 110-kDa *p* locus protein. (*B*) Tyrosinase (detected with αPEP7 antiserum).

gradient. In contrast, when melanized cells (melan-a) were subjected to similar analysis, a shift of a major portion of the 110-kDa protein to dense fractions was observed (Fig. 4*A*), demonstrating that the 110-kDa protein was present in melanin-containing organelles (melanosomes). A more dramatic shift in the density of tyrosinase-rich fractions was observed (Fig. 4*B*). This was in keeping with our observation (S.J.O., P. D. Donatien, and S.R., unpublished observation) that the 110-kDa protein, unlike tyrosinase, cannot be extracted readily from heavily melanized (late stage III–IV) melanosomes.

Processing of the 110-kDa Protein. Many transmembrane proteins are glycosylated, and the amino acid sequence of the p protein reveals four potential N-linked glycosylation sites (4, 5). However, we could not demonstrate the presence of N-linked glycosylation in the 110-kDa *p* locus protein. When melan-a cells were cultured in the presence of tunicamycin, an inhibitor of N-linked protein glycosylation, the molecular mass of TRP-1, an authentic melanosomal membrane glycoprotein, decreased from 75 kDa to 68 kDa, as expected (17) (Fig. 5). In agreement with previously published reports employing the B16 melanoma line (25), treated cells became amelanotic. Levels of LAMP-1, a lysosomal and melanosomal glycoprotein that is rapidly degraded when its glycosylation is inhibited by tunicamycin (26), declined to undetectable levels in cells treated with the highest concentration of drug (Fig. 5). Nonetheless, no effect was observed on the abundance or migration of the 110-kDa protein, even at the highest concentration.

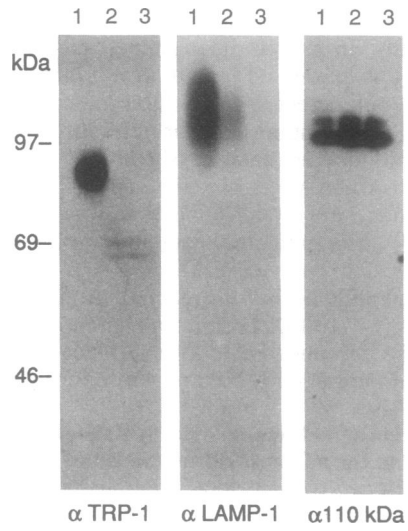

FIG. 5. Tunicamycin treatment does not affect the gel mobility of 110-kDa p protein. The melan-a cells were cultured for 5 days in the absence of tunicamycin (lanes 1) or in the presence of tunicamycin at 0.2 (lanes 2) or 1.0 (lanes 3) μg/ml. Equal quantities of protein from cell extracts were subjected to immunoblot analysis with the indicated antisera. αTRP-1, anti-TRP-1 antiserum; αLAMP-1, anti-lysosome-associated membrane protein 1 monoclonal antibody.

Furthermore, although enzymatic treatment with endoglycosidase F resulted in a shift in the molecular mass of TRP-1 from 75 kDa to 68 kDa, no change in the molecular mass of the *p* locus protein was seen (data not shown).

DISCUSSION

By using antisera directed against the predicted protein product of the *p* locus with appropriate genetic controls, we have identified a 110-kDa integral membrane protein. This protein was present in extracts of both black and albino melanocytes and absent from melanocytes with two independent mutations that result in the functional absence of the mRNA for the *p* gene: p^{cp}, a radiation-induced deletion allele, and *p*, a spontaneous mutation in which *p* transcript levels were reported to be undetectable in the pigmented epithelium and choroid of the eye (4) or to be barely detectable in skin extracts (5). Introduction of the wild-type gene to p^{cp} melanocytes by electroporation resulted in expression of both the 3.3-kb mRNA and the 110-kDa protein, providing further evidence that this protein is indeed encoded at the *p* locus.

Subcellular fractionation studies revealed that the 110-kDa protein localizes to the melanosome-rich LGF and is present in melanin-containing organelles (which, by definition, are melanosomes). Nonetheless, some notable differences in the distribution of the p protein, tyrosinase, and TRP-1 were found. Whereas TRP-1 and tyrosinase are readily detectable in the vesicle-rich small granule fraction, this subcellular compartment lacks the 110-kDa p protein. In addition, while there is substantial overlap in the distribution of tyrosinase and the 110-kDa p protein in albino cells, it is not complete. One possible interpretation of these findings is that, unlike tyrosinase and TRP-1, the p protein is inserted directly into premelanosomes, bypassing the trans-Golgi network–vesicle–melanosome transport route believed to be utilized by the TRP family (for discussion, see refs. 24 and 27).

Although many other proteins with a similar structure, including 12 transmembrane domains, function as membrane channels or transporters of small molecules, to our knowledge, the *p* gene lacks homology to any previously described mammalian gene. While formal proof for a tyrosine transport function for the p protein is lacking, it is of interest that the closest relative of the murine *p* and human *p* genes is a *Mycobacterium leprae* gene encoding a predicted 45-kDa protein (GenBank accession no. L10660). The murine and mycobacterial proteins share a 36.6% identity over a 205-aa stretch. While the function of this latter protein is unknown, it is notable that *M. leprae* is unique among mycobacteria in its ability to take up and oxidize dopa (28, 29).

The p protein may play a structural role within the melanosome. The rapid degradation of the three members of the TRP family in the ocular tissues of mice with a p-null phenotype (13) could be due to a secondary effect of diminished melanosomal tyrosine levels. This degradation could be equally well explained by a structural role played by the p protein. The fact that *p* melanocytes lack the high molecular weight melanogenic complex present in the eyes of wild-type mice (13, 16) lends further support to the structural significance of the protein.

During the course of our studies, we also identified a 65-kDa protein that, by genetic criteria, could not possibly be a product of the *p* locus gene. Nonetheless, three antisera directed against the first cytoplasmic domain of the predicted *p* gene product or a peptide derived therefrom recognize this 65-kDa protein. Furthermore, subcellular fractionation studies suggest that this protein is limited to the 10,000 × *g* supernatant of cultured murine melanocytes. Although the 65-kDa protein is immunologically reactive with antisera raised against the most N-terminal portion of the predicted *p* gene product, further insights into the nature of the 65-kDa protein await molecular cloning of its gene.

Although there are four potential N-glycosylation sites within the predicted p protein, neither tunicamycin treatment nor endoglycosidase F digestion altered the migration of the 110-kDa protein. Despite our inability to demonstrate N-glycosylation within the 110-kDa p protein, we recognize the possibility that it may be a glycoprotein. Our experiments did not rule out the possibility of O-glycosylation of the protein. In addition, if our antiserum recognized only the nonglycosylated precursor of the protein, no effect would be expected upon tunicamycin treatment of cells.

In summary, we have employed molecular, genetic, and immunologic techniques to identify a 110-kDa protein encoded at the *p* locus and an apparently immunologically related but genetically distinct protein of 65 kDa. The 110-kDa protein is a highly hydrophobic integral membrane protein that is localized to the melanosomes. Our results form a framework by which mutations at the *p* loci in mouse and humans that diminish pigmentation can begin to be analyzed at the cellular and biochemical levels.

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