Aberrant pH of Melanosomes in Pink-Eyed Dilution (p) Mutant Melanocytes

Neelu Puri,1 John M. Gardner, and Murray H. Brilliant
Steele Memorial Children’s Research Center, Department of Pediatrics, University of Arizona, Tucson, Arizona, U.S.A.

In past studies, we cloned the mouse p gene and its human homolog P, which is associated with oculocutaneous albinism type 2. Both mouse and human genes are expressed in melanocytes and encode proteins predicted to have 12 membrane-spanning domains with structural homology to known ion transporters. We have also demonstrated that the p protein is localized to the melanosomal membrane and does not function as a tyrosine transporter. In this study, immunohistochemistry and confocal microscopy were used to show that the p protein plays an important role in the generation or maintenance of melanosomal pH. Melanosomes (and their precursor compartments) were defined by antiserum directed against the melanosomal marker tyrosinase related protein 1. Acidic vesicles were identified by 3-(2,4-dinitroanilino)-3'-amino-N-methylidipropylamine incorporation, visualized with anti-dinitrophenol. In C57BL/6+/+ (wild-type) melanocytes, 94.2% of vesicles demonstrated colocalization of tyrosinase related protein 1 and 3-(2,4-dinitroanilino)-3'-amino-N-methylidipropylamine, indicating that almost all melanosomes or their precursors were acidic. By contrast, only 7%-8% of the staining vesicles in p mutant cell lines (p+/p+ and p+/p0) showed colocalization of tyrosinase related protein 1 and 3-(2,4-dinitroanilino)-3'-amino-N-methylidipropylamine. Thus, without a functional p protein, most melanosomes and their precursors are not acidic. As mammalian tyrosinase activity in situ is apparently dependent on low pH, we postulate that in the absence of a low pH environment brought about by ionic transport mediated by the p protein, tyrosinase activity is severely impaired, leading to the minimal production of melanin that is characteristic of p mutants. Additionally (or alternatively), an abnormal pH may also impair the assembly of the normal melanogenic complex. Key words: oculocutaneous albinism/pigmentation/p protein/tyrosinase. J Invest Dermatol 115:607–613, 2000

null mutations of the mouse pink-eyed dilution gene p, and its human homolog P, are defined by hypopigmentation with a near total lack of melanin pigment (Brilliant, 1992; Lyon et al, 1992; Oetting et al, 1996) in melanocytes (Russell, 1949; Markert and Silver, 1956). The reduction of brown/black eumelanin is greater than the reduction in yellow/red pheomelanin (Ozeki et al, 1995; Prota et al, 1995). Previously, we cloned the p gene and found it to encode a protein with 12 predicted membrane-spanning domains (Gardner et al, 1992). From this predicted protein structure and the phenotype of melanocytes with p gene mutation, we (Gardner et al, 1992; Rosembalt et al, 1994) and others (Rinchik et al, 1993; Lee et al, 1994; 1995) hypothesized that the p gene is a transport or pore protein critical to melanosomal function. Our initial results with antibodies against the p protein demonstrated that the p protein is associated with the melanosomal membrane (Rosembalt et al, 1994). Therefore, the p protein might transport a critical substance between the cyttoplasm and melanosomes. Sidman and Pearlstein (1965) observed that retinal melanocytes (in organ culture) from p/p mice become melanized in the presence of high concentrations of tyrosine, a precursor for melanin. They speculated that the p protein could be involved in tyrosine uptake or otherwise modulate tyrosinase activity due to an effect on tyrosine-utilizing systems. To assay whether or not the p protein was involved in tyrosine transport, we (Gahl et al, 1995) and others (Potter et al, 1998) measured tyrosine transport across the cell and melanosomal membranes. The results of those studies indicated that tyrosine transport was the same in both wild-type and homozygous p mutant melanocytes. Thus, tyrosine transport is not mediated by the p protein.

The initial and rate limiting step in melanin biosynthesis is catalyzed by tyrosinase (reviewed by Pawelek and Chakraborty, 1998). In addition to the substrate tyrosine, maximal in situ tyrosinase activity requires an appropriate ionic environment. The melanosomal lumen is known to be acidic (Moellmann et al, 1988; Bhatnagar et al, 1993). Devi et al (1987) have shown that preincubation of murine tyrosinase at an acidic pH causes the enzyme to lose the lag period that occurs when tyrosine is a substrate [in the absence of added L-dihydroxyphenylalanine (L-DOPA)]. Thus, the acidic pH of melanosomes favors optimum tyrosinase activity and the melanization of melanosomes (Ramaiyah, 1996). In this study, we tested the hypothesis that the p protein functions in the acidification of melanosomes, presumably as an ion exchange (or channel) protein in the melanosomal membrane. To test this hypothesis, we assayed the pH of melanosomes and their
precursors from both wild-type and p mutant melanocytes in vitro. Melanosomal and premelanosomal compartments were identified by the presence of the melanosomal marker, tyrosinase related protein 1 (Tyrp1) (Vijayasaradhi et al., 1991). Acidic compartments were identified using 3-2,4-dinitrophenol)-3'-amino-N-methyl-dipropylamine (DAMP, a basic congener of dinitrophenol that accumulates in acidic components, where it can be fixed in situ with aldehydes)). DAMP was used in studies by Anderson and Pathak (1985) to demonstrate the acidic nature of the lysosome, an organelle related to melanosomes (Orlow, 1995). The results of this study implicate involvement of the p protein in the generation or maintenance of an acidic melanosomal pH.

MATERIALS AND METHODS

Establishment of melanocyte cultures from neonatal mice The dorsal skin of a neonatal mouse was removed asepically and cut into several small pieces, rinsed in Ca2+/Mg2+-free phosphate-buffered saline (PBS), and incubated in 0.25% trypsin at 37°C and 5% CO2 for 2 h. The demarcation was then separated. 100 U per ml of penicillin, 100 mg/ml of streptomycin, 2 mM L-glutamine, 11.1 mM Na2HPO4, 1.1 mM d-butyric cyclic adenosine-5-monophosphate, 100 mM phorbol-12-myristate-13-acetate and 25 μg per ml bovine pituitary extract-protein (Life Technologies, Rockville, MD). Melanocyte lines from C57BL/6/+ or +/+ or +/+ were established from primary cultures. The last two cell lines are null for p gene function. The p allele is characterized by an intragenic deletion (Oakey et al., 1996) and pF/pF is a compound heterozygote of two deletion alleles and lacks all protein encoding sequences of the p gene (Gardner et al., 1992; Nakatsu et al., 1993; Lehman et al., 1998). After establishing pure cultures of these melanocytes, electron microscopy and immunofluorescent studies were performed, comparing cells of the same passages (passages 3-6).

Electron microscopy Confluent flasks of melanocytes were treated with 0.05% trypsin and 0.02% EDTA in Ca2+/Mg2+-free PBS, pelleted, and washed twice in PBS. Cell pellets were fixed for at least 18 h at 4°C in 3% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2). The fixed pellets were dissected into 1 mm3 pieces. Postfixation was in 1% osmic tetroxide in 0.1 M phosphate buffer for 1 h followed by dehydration in 30 min in 1% uranyl acetate in 50% ethanol. The tissues were then dehydrated using serial alcohol and aceone incubations and embedded in Spurr resin. A Sorvall MT-2B ultramicrotome was used to section the tissues to 80 nm (silver-gold). Sections were stained with uranyl acetate and lead citrate. Grids were viewed on a Philips 400 electron microscope at an accelerating voltage of 80 kV.

Tyrosinase activity To visualize tyrosinase activity in melanocytes, L-DOPA conversion to melanin was assayed in vitro. Tyrosinized cell pellets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, and then washed three times in cacodylate buffer. These fixed pellets were dissected into 1 mm3 pieces and incubated in 0.1% L-DOPA in cacodylate buffer at 37°C for 1 h. After incubation, the samples were washed three times with 0.1% L-DOPA cacodylate buffer at room temperature for 2 h, and then incubated a third time in 0.1% L-DOPA cacodylate buffer at 37°C for 2 h. Postfixation was done in 1% osmic tetroxide in 0.1 M cacodylate buffer, and all further steps of electron microscopy processing were performed as described above.

Immunofluorescent studies of mouse melanocytes using confocal laser scanning microscopy Melanocytes (1 x 105) from pF/pF or pF/pO mice were plated on glass cover slips in six-well plates. Cells were allowed to grow for 48 h at 37°C, and then were incubated with 30 μM DAMP for 30 min at 37°C and washed with Ham’s F10 medium. For monoclonal antibodies, cells were fixed with 25 μg/ml of (Sigma Chemical, St. Louis, MO) for 5 min after DAMP treatment. To localize DAMP, cells were fixed at room temperature for 15 min in 3% (wt/vol) parafomaldehyde in buffer A (10 mM sodium phosphate, 150 mM sodium chloride, 2 mM magnesium chloride; pH 7.4), and then were washed once with 50 mM ammonium chloride and twice with buffer A. Each monolayer was permeabilized with 0.1% (vol/vol) Triton X-100 in buffer A for 5 min at -10°C. Coverglasses were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 30 min. They were then covered with 100 μl of monoclonal mouse anti-dinitrophenol (DNP) IgG diluted 1:10 (Oxford Biomedical Research) and incubated at 37°C for 60 min. After washing with buffer A, the cells were incubated with 100 μl fluorescein-5-isothiocyanate (FITC) conjugated goat antimouse IgG diluted 1:200 (Organon Teknika Corporation) at 37°C for 60 min. After three additional washes with buffer A, the melanocytes were blocked again with 5% goat serum for 30 min and incubated with rabbit polyclonal antisera PEPI diluted 1:500 (a kind gift of V. Nair, NIH) generated against the carboxy-terminal sequence of mouse Tyrp1 (Janene et al., 1991) for 1 h at room temperature. Coverslips were washed 3 times in buffer A, incubated with lissamine rhodamine-conjugated goat antirabbit IgG diluted 1:100 (Jackson ImmunoResearch Laboratories) for 60 min and washed with buffer A. These coverslips were then mounted in Vecta shield (Vector Laboratories, Burlingham, CA) and viewed under a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad, Richmond, CA) equipped with an argon krypton laser coupled to a Nikon Optiphot II fluorescence microscope and a 60 X plan apo oil objective. A standard k1/k2 filter set was used. Simultaneous two-channel recording was performed using excitation wavelengths of 488 and 568 nm. Images were processed and merged using a Voel view ultra 2.5 software (Vital Images, Airfield, IA).

The numbers of vesicles that stained for Tyrp1, DAMP, or both were scored in four independent experiments. In each experiment, 25 independent fields (47 μm2) were counted from cells established from C57BL/6/+ or +/+ and the p mutants. The data was obtained from (4 X 25) representative fields of approximately equivalent vesicular densities of peripheral cytoplasm were used to calculate the percentage of vesicles stained with Tyrp1 and DAMP in all three cell lines.

Immunogold studies of wild-type melanocytes C57BL/6 wild-type melanocytes were grown and treated with DAMP as detailed above, and pelleted in Ca2+/Mg2+-free PBS, pH 7.2. The cells were then fixed for 1 h in 1% glutaraldehyde in Ca2+/Mg2+-free PBS, pH 7.2, and washed for 5 min three times in Ca2+/Mg2+-free PBS. The remaining glutaraldehyde was quenched by incubation in 0.1 M NH4Cl in Ca2+/Mg2+-free PBS, pH 7.2, for 30 min, followed by two 5 min washes in Ca2+/Mg2+-free PBS, pH 7.2. The treated cell pellets were then dehydrated by serial alcohol dilution and embedded in LRWhite at 50°C, under vacuum. The cell pellets were cut in 80 nm sections onto 200 mesh formvar/carbon coated nickel grids. The grids were floated on a drop of blocking buffer (0.5 M NaCl, 0.1% Na-azide, 1% ovalbumin, in 0.01 M Tris-HCl, pH 7.2) for 30 min at room temperature. The grids were then floated on a drop of blocking buffer plus mouse monoclonal anti-DNP (Oxford Biomedical Research) at 1:10 dilution for 16 h at 4°C, in a moist chamber. The grids were then washed with rinse buffer (0.15 M NaCl in 0.01 M Tris-HCl, pH 7.2) and floated on rinse buffer plus 0.02% polyethylene glycol-20, 0.1% Na-azide, plus Protein A gold, 10 nm (Amerham), at 1:50, for 1 h at room temperature and washed three times with rinse buffer. Fixation of the antigen-antibody complex was with 2% glutaraldehyde in PBS for 10 min followed by two washes with ddH2O. The grids were then stained with 2% uranyl acetate (aqueous) for 10 min and lead citrate for 10 min and viewed on a Philips 400 electron microscope at 80 kV.

RESULTS

Electron microscopy studies with melanocytes from C57BL/6/+ and p mutant mice Cultures of melanocytes were established from C57BL/6/+ or +/+ or +/+ mice, both p null mutant cell lines exhibited minimal amounts of visible pigment in their cell pellets or culture media. Electron microscopy demonstrated that melanocytes from C57BL/6/+ (Fig 1A) contained numerous melanosomes that were poorly melanized (stage I-II), while melanocytes from pF/pF mice were highly melanized (stage III-IV). Melanocytes from p null mutants also contained numerous melanosomes, but these were poorly melanized (stage I-II) (Fig 1B, c); similar results were obtained by Rosenblat et al. (1998). In addition, we tested L-DOPA reactivity of the pF/pO melanocytes that we established in vitro. These melanocytes exhibited L-DOPA oxidase activity of tyrosinase that revealed the presence of this enzyme in the trans-Golgi network and in stage I-II melanosomes (Fig 1D).

Immunofluorescent studies using confocal laser scanning microscopy Normal melanocytes have been shown to be acidic organelles (Moellman et al., 1998; Bhatnagar et al., 1993). To determine whether melanosomes from p mutant melanocytes are
acetic, we used DAMP and anti-DNP staining to visualize acidic organelles. Anderson et al. (1984) have shown that DAMP penetrates the membranes of living cells, accumulates in acidic vesicles within the cell, and can be retained in these vesicles after aldehyde fixation. Melanocytes from C57BL/6+/+ and p mutant mice were treated with DAMP, fixed in paraformaldehyde, and processed for indirect immunofluorescence (Fig. 2) using mouse monoclonal antibodies directed against dinitrophenol (anti-DNP) and visualized with goat antimouse antibodies coupled to FITC. The polyclonal antibody PEP1 directed against Tyrp1 was used to detect melanosomes and their precursors and visualized with goat antirabbit IgG conjugated with lissamine rhodamine.

Tyrp1-staining vesicles were detected primarily in the perinuclear area but were also scattered throughout the cytoplasm in melanocytes from all three genotypes (Fig. 2b, e, h). To ensure that our assay for DAMP accumulation and binding was specific for acidic vesicles, we included control experiments employing monensin treatment of melanocytes from both C57BL/6+/+ and p mutants. The carboxylic ionophore monensin is known to dissipate proton gradients by exchanging protons for potassium ions across membranes (Pressman, 1976). Melanocytes from all genotypes were allowed to take up DAMP, and were then washed and exposed to monensin for 5 min. After monensin treatment, DAMP was no longer visualized within the cells (Fig. 2j, k). These results confirm that in our assays the accumulation of DAMP and its retention in cell organelles is dependent on a pH gradient across the membrane of these organelles.

To determine the number of vesicles that stained for Tyrp1, DAMP, or both, we counted a total of 100 fields of 47 μm² (25 fields in each of four independent experiments) of peripheral cytoplasm. A total of 7846 vesicles were counted. The average number of stained vesicles per 47 μm² field was 22.4 in C57BL/6+/+, 27.9 in p+/p⁺; 28.1 in p+/p⁻. The three cell lines contained roughly the same number of Tyrp1-staining vesicles per single 47 μm² field (C57BL/6+/+, 20.0; p+/p⁺, 18.4; p+/p⁻, 19.1). In C57BL/6+/+ melanocytes 94.2% of melanosomes defined by Tyrp1 staining were also acidic by DAMP staining (i.e., colocalization of Tyrp1 and DAMP, Fig. 2c, Table 1). In melanocytes from p+/p⁺ and p+/p⁻ mice, however, only 7.4% and 8.4%, respectively, of the Tyrp1-staining vesicles exhibited colocalization with DAMP (Fig. 2f, i, Table 1).

To confirm that the acidic vesicles visualized by DAMP staining were indeed melanosomes, DAMP-staining vesicles were revealed by electron microscopy using immuno-gold conjugated anti-DNP. As shown in Fig. 3, structurally identifiable melanosomes in wild-type melanocytes were clearly stained in this assay. Early stage to mature melanosomes were stained, indicating that melanosomes are acidic even before melanin synthesis and deposition.

We also found a class of organelles that were acidic (DAMP staining) but were not melanosomes (not Tyrp1 staining) in wild-type and mutant cell lines. These acidic nonmelanosomal vesicles accounted for about 11% of the vesicles detected in wild type and for about 33% of the vesicles detected in the two mutant cell lines (Table 1). In addition, the mutant cell lines appear to have a 25% increase in the total number of staining vesicles per field (Table 1).
These extra vesicles seen in mutant cell lines were primarily nonmelanosomal acidic vesicles and were not further characterized in this study.

**DISCUSSION**

We have established melanocyte cell lines that recapitulate the *in vivo* phenotypes of melanocytes from wild-type and *p* null mutant mice. Electron microscopy revealed mature and highly pigmented stage IV melanosomes in the wild-type cell line, whereas only immature stage I and stage II melanosomes were detected in the *p* null mutant cell lines, although the mutant melanosomes did possess tyrosinase activity using L-DOPA as substrate (Fig 1). Similar observations have been made *in vivo* (Orlow and Brilliant, 1999) and *in vitro* (Potterf et al, 1998; Rosenblat et al, 1998). Like their wild-type derived counterparts, the *p* null derived melanocytes also possess Tyrp1 staining melanosomes and their precursors (Fig 2).

To evaluate the possible role of the *p* protein in the acidification of melanosomes, we characterized the intracellular compartments of melanocyte cell lines derived from wild-type mice and two *p* mutants, *p*/*p* and *p*/*p*/*p*. We stained acidic compartments using DAMP, a basic congener of DNP. DAMP has a primary and tertiary amino group that becomes protonated and positively charged at acidic pH. The primary amino group allows DAMP to become covalently linked to proteins in the presence of aldehyde fixatives, such as formaldehyde, which allows it to be retained in acidic organelles after fixation (Anderson et al, 1984; Anderson and Pathak, 1985). DAMP can be visualized after fixation with appropriately tagged anti-DNP antibodies via a dinitroanene group that reacts with monoclonal antibodies to DNP. Control experiments were conducted using the ionophore monensin that disrupts the proton gradient by exchanging protons for potassium across the membrane. No DAMP was detected in the melanocytes after monensin treatment indicating that DAMP retention is dependent on a pH gradient across vesicular membranes. Our data demonstrate that in C57BL/6×/× melanocytes the major class of acidic organelles contains Tyrp1 that presumably includes melanosomes and their precursors. These results confirm the earlier results of Moellman et al (1988) who used DAMP to demonstrate that the pH of the melanosomes is acidic. Using other pH indicators, Bhatnag et al (1993) determined the pH of the melanosome to be in the range 3.0–4.6 and found that the pH of this organelle is inversely correlated with its degree of melanization. Our results indicated that the vast majority (94.2%) of melanosomes and their precursors from the C57BL/6×/× melanocyte cell line were acidic (i.e., showed colocalization of Tyrp1 and DAMP). In two *p* deficient mutant melanocyte lines derived from *p*/*p*/*p* and *p*/*p*/*p* mice, however, only 7.4% and 8.4%, respectively, of melanosomes were found to be acidic (Fig 2, Table 1). Thus, the major difference between the wild-type and *p* mutant melanocyte cell lines is the near absence of acidic melanosomes in *p* mutant melanocytes. This observation suggests that the *p* protein plays an important role in the acidification of melanosomes.

**Figure 2.** Confocal laser scanning microscopy of dermal melanocytes from C57BL/6×/× and from *p* mutant mice (*p*/*p* and *p*/*p*/*p*) immunostained with antibodies against DAMP and Tyrp1. Acidic compartments of melanocytes were detected by DAMP incorporation followed by treatment with anti-DNP antiserum; melanosomes were detected by anti-Tyrp1 antiserum. Parts (a)–(c) show C57BL/6×/× melanocytes (a) stained with anti-DNP antibodies visualized as green fluorescence; (b) stained with anti-Tyrp1 antibody visualized as red fluorescence; (c) stained with both anti-DNP and anti-Tyrp1 antiserum (merged image), demonstrating extensive colocalization of acidic compartments and melanosomes visualized as yellow fluorescence. Parts (d)–(f) and (g)–(i) are similarly stained and are from melanocytes from *p*/*p*/*p* and *p*/*p* mice, respectively. Melanosomes from *p* mutant mice show very little colocalization of DAMP and Tyrp1. Part (j) shows a merged image of C57BL/6×/× melanocytes treated with 25 μm monensin for 5 min after DAMP incorporation, followed by immunostaining with DAMP and Tyrp1 antibodies. The latter control demonstrates the loss of acidic compartments after monensin treatment. Part (k) is a merged image of melanocytes from *p*/*p*/*p* mice similarly treated as in (j). All panels are reproduced at the same magnification. Scale bar: 13.8 μm.
Table I. Percentage of organelles in wild-type and p mutant melanocytes that are melanosomes (defined by Tyrp1 staining),
acidic vesicles (defined by DAMP staining), or both

<table>
<thead>
<tr>
<th>Source</th>
<th>Melanosomes</th>
<th>Non-melanosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Tyrp1 + DAMP</td>
<td>+ Tyrp1 − DAMP</td>
</tr>
<tr>
<td></td>
<td>(yellow fluorescence)</td>
<td>(red fluorescence)</td>
</tr>
<tr>
<td></td>
<td>number of vesicles;%</td>
<td>number of vesicles;%</td>
</tr>
<tr>
<td>C57/BL6**</td>
<td>457; 82.3%</td>
<td>29; 5.3%</td>
</tr>
<tr>
<td>I.</td>
<td>483; 82.8%</td>
<td>30; 5.1%</td>
</tr>
<tr>
<td>II.</td>
<td>481; 84.5%</td>
<td>30; 5.3%</td>
</tr>
<tr>
<td>IV.</td>
<td>467; 86.6%</td>
<td>27; 4.8%</td>
</tr>
<tr>
<td>total vesicles</td>
<td>840 ± 1.7%</td>
<td>5.1 ± 0.2%</td>
</tr>
<tr>
<td>melanosomes</td>
<td>94.2 ± 0.3%</td>
<td>5.7 ± 0.3%</td>
</tr>
</tbody>
</table>

*p*/pH

<table>
<thead>
<tr>
<th>Source</th>
<th>Melanosomes</th>
<th>Non-melanosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Tyrp1 + DAMP</td>
<td>+ Tyrp1 − DAMP</td>
</tr>
<tr>
<td></td>
<td>(yellow fluorescence)</td>
<td>(red fluorescence)</td>
</tr>
<tr>
<td></td>
<td>number of vesicles;%</td>
<td>number of vesicles;%</td>
</tr>
<tr>
<td>I.</td>
<td>25; 3.5%</td>
<td>403; 57.0%</td>
</tr>
<tr>
<td>II.</td>
<td>48; 6.9%</td>
<td>410; 59.2%</td>
</tr>
<tr>
<td>III.</td>
<td>36; 5.0%</td>
<td>463; 64.2%</td>
</tr>
<tr>
<td>IV.</td>
<td>27; 4.0%</td>
<td>423; 63.0%</td>
</tr>
<tr>
<td>total vesicles</td>
<td>4.8 ± 1.3%</td>
<td>60.8 ± 2.9%</td>
</tr>
<tr>
<td>melanosomes</td>
<td>7.4 ± 1.9%</td>
<td>92.6 ± 1.9%</td>
</tr>
</tbody>
</table>

*p*/p/

<table>
<thead>
<tr>
<th>Source</th>
<th>Melanosomes</th>
<th>Non-melanosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Tyrp1 + DAMP</td>
<td>+ Tyrp1 − DAMP</td>
</tr>
<tr>
<td></td>
<td>(yellow fluorescence)</td>
<td>(red fluorescence)</td>
</tr>
<tr>
<td></td>
<td>number of vesicles;%</td>
<td>number of vesicles;%</td>
</tr>
<tr>
<td>I.</td>
<td>30; 4.3%</td>
<td>440; 63.4%</td>
</tr>
<tr>
<td>II.</td>
<td>51; 7.2%</td>
<td>428; 60.1%</td>
</tr>
<tr>
<td>III.</td>
<td>47; 6.5%</td>
<td>467; 64.6%</td>
</tr>
<tr>
<td>IV.</td>
<td>33; 4.9%</td>
<td>418; 61.6%</td>
</tr>
<tr>
<td>total vesicles</td>
<td>5.7 ± 1.2%</td>
<td>62.4 ± 1.7%</td>
</tr>
<tr>
<td>melanosomes</td>
<td>8.4 ± 1.7%</td>
<td>91.6 ± 1.7%</td>
</tr>
</tbody>
</table>

*Tyrp1 staining vesicles only. The identity of the non-Tyrp1 staining acidic vesicles is unknown, among the possibilities are very early (non-Tyrp1 expressing) melanosomal precursors or other acidic endosomal-derived vesicles. In each of the four sets of experiments (I-IV), 25 independent fields of 47 μm² of peripheral melanocyte cytoplasm were evaluated (see Materials and Methods). The data summarized for each cell line (in bold) represent the percentages of each vesicle type (mean values ± standard deviation) in all four experiments, i.e., 100 independent fields of peripheral cytoplasm of similar vesicular densities.

We found more acidic vesicles in the two mutant p melanocyte lines than in the single wild-type line examined (Table I), possibly reflecting independent variables (genetic or in vitro) between these three cell lines. Alternatively, this may underscore an, as yet unknown, compensatory mechanism. One hypothesis is that these acidic nonmelanosomal vesicles in the mutant cells represent increased numbers of lysosomes that are required to degrade the resulting nonfunctional melanosome components.

Mutations in the P gene, the human homolog of the mouse p gene, have been shown to be responsible for ocucutaneous albinism type 2 or OCA2 (Gardner et al., 1992; Durham-Pierre et al., 1994; Rinchik et al., 1993). Both the mouse and human genes encode a protein with 12 membrane-spanning domains (Gardner et al., 1992; Rinchik et al., 1993) localized to the melanosome membrane (Rosenblat et al., 1994). With its 12 membrane-spanning domains, the p protein shares significant homology to known protein transporters (Gardner et al., 1992; Rinchik et al., 1993), in particular anion transporters (Lee et al., 1995).

Tyrasinase catalyzes the first and rate limiting enzymatic reaction in the production of melanin from the precursor tyrosine (reviewed by Pawelek and Chakraborty, 1998). Tyrasinase is associated with the melanosomal membrane (Potterf et al., 1998) and its activity is increased under acidic conditions (Ramaiah, 1996). In this context there are two potential roles for a transporter protein: tyrosine transport or ionic transport to regulate melanosomal pH. Sidman and Pearlstein (1965) were the first to suggest that the p protein might play a role in the transport of tyrosine. In previous studies, however, we (Gahl et al., 1995) found that the characteristics of tyrosine transport in normal melanocytes were indistinguishable from melanocytes from homozygous p mutants. This result has been recently confirmed in other studies (Potterf et al., 1998). These studies show that the p protein is not a tyrosine transporter. The p protein could be an ion transporter that is involved in the process of acidification of melanosomes, however.

Melanosomes and lysosomes may be products of a common (endosomal) intracellular pathway, and both types of organelles are characterized by an acidic lumen (Orlow, 1995). The identity of the molecular components responsible for maintenance of the acidic environment of endosomes is not known, but it is thought that an anion channel and an adenosine-5′-triphosphate (ATP)-driven proton pump are essential components in the acidification of endosomes. Anion (e.g., Cl⁻ or PO₄³⁻) conductance provides the compensating charge balance to electrogenic proton transport (Al-Awqati, 1995; Van Dyke, 1996). It is possible that the p protein is an anion transporter based on its sequence similarity to known anion transporters (Chen et al., 1986; Lee et al., 1995; Bun-ya et al., 1996) involved in arsenate resistance in bacteria and PO₄³⁻ transport in yeast. Like lysosomes, melanosomes have been shown to contain proton-translocating ATPase that may be involved in their acidification (Bhatnagar et al., 1993; Orlow, 1995). The absence of this (melanosomal) proton-translocating ATPase could result in melanosomes that are not acidic. It is unlikely, however, that the p protein is this melanosomal proton–translocating ATPase, as known ATPases do not share a similar protein structure (i.e., 12 membrane-spanning domains). Thus, although the specificity of the proposed transport function of the p protein remains to be determined, the protein homology data, combined with our results, strongly suggest that the p protein is a transporter involved in melanosomal acidification. As an almost exclusively melanocyte-specific gene product (Gardner et al., 1992), it is conceivable that the p protein is part of a specialized system required to acidify melanosomes that is distinct from the system used to acidify lysosomes. For example, inclusion of the p protein in its membranes may commit an endosomal vesicle to becoming a melanosome through specific protein targeting or melanosome biogenesis, in addition to providing an acidic environment favorable for optimum tyrosinase function.

Mammalian tyrosinase activity in vivo is inversely related to pH and the kinetics of tyrosinase activity in vitro is also affected...
by pH with a significant decrease in lag time at more acidic pH (Bhattachar et al, 1993; Ramaiah, 1996). Thus, in the absence of p-protein-mediated acidification of the melanosome, it is very likely that there is a decrease in tyrosinase activity in vivo. This alone could result in a minimal amount of melanin produced in p mutants. Intriguingly, we have also observed that under low pH conditions p melanogenesis grown in vitro make more melanin (unpublished observations). Tyrosinase exhibits Michaelis-Menten kinetics (Laskin and Piccinini, 1986). As such, an increase in substrate can increase the initial velocity of the reaction (i.e., μM of product per min per mg of protein) even under suboptimal (nonacidic) conditions. This seems to be true in the case of melanin production mediated by tyrosinase in p mutant melanosomes. Following exposure of p mutant melanocytes to high (above physiologic) concentrations of tyrosine in vitro, there is an increase in melanin and late stage melanosomes (stage III–IV) that are almost normal in size (Sidman and Pearlstein, 1965; Rosemblat et al., 1994, 1998; Poterf et al., 1998).

It is also possible that the p protein may play a role in melanosome biogenesis by contributing to luminal acidification in developing melanosomes. In normal pigmented melanocytes, a fraction of the p protein is present in an intracellular compartment distinct from those containing tyrosinase and Typr1 (Rosemblat et al., 1994). It has also been noted that melanosomes lacking p protein are missing a high molecular weight complex of the p protein, tyrosinase, and Typr1, and possess characteristics of immature premelanosomes (Rosemblat et al., 1994; 1998). Moreover, tyrosinase and Typr1 localization to melanosomes, mediated by peptide sorting signals (Vijayasaradhi et al., 1995), may be important in p mutant melanosomes (Poterf et al., 1998).

Although the exact biochemical function of the p protein remains to be elucidated, our data indicate that it clearly plays a role in the acidification of melanosomes that may be direct or indirect, affecting the activity and/or routing of the rate limiting enzyme, tyrosinase.

The authors wish to thank Tracey Gales and Manfred Bayer for electron microscopy studies, Jonathan Boyd for confocal studies, Canole Meyer for manuscript preparation, and Vince Hearing for generously providing the PEPI antisem. We thank Fayez Ghiash, Setaluri Vijayasaradhi, J. Newton, Nobuko Hagiwara, and Richard Geuweke for helpful comments on the manuscript. This work was supported by NIH/NIAMS grant RO1 AR 45496 to (M.H.B.).

REFERENCES

Oakey RJ, Keiper NM, Ching AS, Brilliant MH: Molecular analysis of the CNDA-encoded by the p<sup>+</sup> and p<sup>+</sup> alleles of the pink-eyed dilution locus. Mammalian Genome 7:315–316, 1996
Ozeki H, Ito S, Watanabe K, Hirobe T: Chemical characterization of hair...
Russell ES: A quantitative histological study of the pigment found in coat colour mutants of the house-mouse. The nature of genetic effects of five major allelic series. Genetics 34:146–166, 1949