Reduced pigmentation (rp), a mouse model of Hermansky-Pudlak syndrome, encodes a novel component of the BLOC-1 complex


Hermansky-Pudlak syndrome (HPS), a disorder of organelle biogenesis, affects lysosomes, melanosomes, and platelet dense bodies. Seven genes cause HPS in humans (HPS1-HPS7) and at least 15 nonallelic mutations cause HPS in mice. Where their function is known, the HPS proteins participate in protein trafficking and vesicle docking/fusion events during organelle biogenesis. HPS-associated genes participate in at least 4 distinct protein complexes: the adaptor complex AP-3; biogenesis of lysosome-related organelles complex 1 (BLOC-1), consisting of 4 HPS proteins (pallidin, muted, cappuccino, HPS7/sandy); BLOC-2, consisting of HPS6/ruby-eye, HPS5/ruby-eye-2, and HPS3/cocoa; and BLOC-3, consisting of HPS1/pale ear and HPS4/light ear. Here, we report the cloning of the mouse HPS mutation reduced pigmentation (rp). We show that the wild-type rp gene encodes a novel, widely expressed 195-amino acid protein that shares 87% amino acid identity with its human orthologue and localizes to punctate cytoplasmic structures.

Introduction

Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder of organelle biogenesis that affects lysosomes and 2 lysosome-related organelles (LROs), melanosomes and platelet dense bodies. HPS occurs in all ethnic groups but is particularly prevalent in northwest Puerto Rico (1/1800) due to a founder effect involving HPS1. Melanosome defects lead to oculocutaneous albinism causing nystagmus, decreased visual acuity, and skin damage on exposure to the sun. Platelet dense body defects result in prolonged bleeding, which can be severe. Some patients develop progressive pulmonary fibrotic disease, which typically leads to death in the fourth to fifth decades. The pathogenesis of lung fibrosis is poorly understood. It has been attributed to ceroid lipofuscin accumulation in defective lysosomes within alveolar macrophages. Recent studies in mice suggest that a defect in a fourth LRO, lamellar bodies of type II pneumocytes, may contribute to lung disease in HPS.

HPS is clinically and genetically heterogeneous. Defects in 7 genes (HPS1-HPS7) cause HPS in humans. Patients with HPS-1 and HPS-4 are at high risk for pulmonary involvement. At least 15 nonallelic mutations cause HPS in mice, including the orthologues of HPS1-HPS7: pale ear (pe), pearl (pe), cocoa (coa), light ear (le), ruby-eye-2 (ru2), ruby-eye (ru), and sandy (sdv), respectively. Mutations at 6 loci in mice have been cloned for which no human HPS patients have yet been identified: mocha (mh), gunmetal (gm), buff (bf), pallid (pa), muted (mu), and cappuccino (cno).

Where their function is known, the HPS proteins participate in sorting of cargo proteins and vesicle targeting/fusion events during the biogenesis of LROs. Genes encoding the β3A (Ap3b1) and δ (Ap3d) subunits of the adaptor complex AP-3, which functions in endosomal-lysosomal protein trafficking, are defective in HPS-2/ pearl and mocha, respectively. Buff is a defect in the gene encoding VPS33A, which is involved in protein transport to lysosomes in mammals as well as the yeast vacuole. pallid is a defect in a novel protein, pallidin, which binds to syntaxin-13, a t-SNARE protein that mediates vesicle docking and fusion. Sandy encodes dysbindin (Dnmlp1), which binds to pallidin in addition to components of the dystrophin-associated protein complex. In gunmetal, the gene encoding the α subunit of Rab geranylgeranyl transferase (Rabgga) is mutated; rab proteins function in targeting and fusing transport vesicles to their acceptor membranes.

The remaining HPS genes encode novel proteins containing no functional domains or structural motifs that give any clues as to their precise function. However, the phenotypic similarity among the mouse HPS models clearly points to a role in some aspect of organelle biogenesis. Moreover, the HPS proteins function within...
multiple subunit complexes. HPS2/pe and mocha are components of the AP-3 complex.27 Muted, pallidin, cappuccino, HPS7/sdy, and at least one additional unknown protein interact in a complex designated BLOC-1 (biogenesis of lysosome-related organelles complex 1).5,21,28 The presence of the syntaxin-binding protein palladin suggests that BLOC-1 is involved in vesicle docking and fusion. HPS5/tn2, HPS6/tnu, and HPS3/coa are components of BLOC-2,16,29 whereas HPS1/ep interacts with HPS4/le in BLOC-3.20,31 Interestingly, unlike AP-3, the known components of BLOC-1, -2, and -3 are confined to metazoans, suggesting they serve specialized functions in the biogenesis of those LROs (eg, platelet dense bodies, melanosomes) that are specific to higher eukaryotes.

Of the known HPS mouse models, only subtle gray (sut)32 and reduced pigmentation (rp)33 have not been cloned. Here, we report the positional cloning of reduced pigmentation (GenBank accession no. AY515509) and show that wild-type rp encodes a novel, ubiquitously expressed 195-amino acid protein that in its phosphorylated form is part of the BLOC-1 complex. In mutant rp/rp mice a premature stop codon truncates the protein at 79 amino acids indicating that failure to produce an intact BLOC-1 complex underlies the HPS phenotype in rp/rp mice. Defects in the other known components of BLOC-1 (sdy, mu, pa, cno) all cause HPS as well, suggesting that these subunits are nonredundant and play key roles in the biogenesis of LROs.

Patients, materials, and methods

Animals

All animals were raised at The Jackson Laboratory, Bar Harbor, ME. The autosomal recessive rp mutation arose spontaneously on the C57BL/Tb (Tb) background in 1975.33 The mutation was back-crossed to C57BL/10ScSn (B10) for 2 generations and subsequently maintained on the Tb and B10 mixed genetic background by rp/+ × rp/rp matings. Heterozygotes, which show no phenotype, and closely related C57BL/6 (B6) mice served as normal controls in all studies. All animals were housed in humidity- and temperature-controlled rooms with a 12-hour light cycle with free access to acidified water and chow (NIH 5K52). The Jackson Laboratory Animal Care and Use Committee approved all protocols.

Blood analysis

Adult whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated microtainer tubes (Becton Dickinson, Rutherford, NJ). Complete blood counts were obtained using an Advia 120 multispecies whole blood analyzer ( Bayer, Tarrytown, NY) as described.34 Platelet dense bodies were enumerated in air-dried, unstained, unfixed whole platelets as described35 using a JEOL 100CXII transmission electron microscope (Jeol, Tokyo, Japan). Whole blood was collected in acid-citrate-dextrose (0.13 M citric acid, 0.15 M sodium citrate, 0.1 M dextrose) and centrifuged at 120 minutes at room temperature to obtain platelet-rich plasma. Bleeding times were measured from tail tissue using a commercial kit (Gentra Systems, Minneapolis, MN). Oligonucleotides were designed complementary to sequence publicly available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and were synthesized by Ransom Hill Bioscience (Ramona, CA). Sequencing was performed using the automated dye termination technique (ABI PRISM Model 3700 Genetic Analyzer, Applied Biosystems, Foster City, CA). Sequence data were obtained using the Applied Biosystems BigDye Terminator Kit 1.1 (Applied Biosystems, Foster City, CA) and analyzed using Seqscape software (Life Technologies, Carlsbad, CA). Sequences were deposited in GenBank (B762177 to B762187).

Sequencing

gDNA was prepared from spleens of C57BL/6J (B6) and rp/rp mice. Approximately 1000 bp fragments within the rp critical interval on chromosome 7 and overlapping by 50 to 200 bp at either end were generated by PCR using high-fidelity Dynazyme EXT DNA polymerase according to the manufacturer’s instructions (MJ Research, Watertown, MA). DNA fragments were sized by electrophoresis in 0.8% agarose gels, excised from the gel, and purified using a Spin-X column (Corning Costar, Corning, NY). Oligonucleotides were designed complimentary to sequences publicly available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and were synthesized by Ransom Hill Bioscience (Ramona, CA). Sequencing was performed using the automated dye termination technique (ABI PRISM Model 3700 Genetic Analyzer, Applied Biosystems, Foster City, CA). Sequence data was obtained using the Applied Biosystems BigDye Terminator Kit 1.1 (Applied Biosystems, Foster City, CA) and analyzed using Seqscape software (Life Technologies, Carlsbad, CA). Sequences were deposited in GenBank (B762177 to B762187).

Northern and Southern blotting

Total RNA was prepared using TRizol reagent (Invitrogen, Carlsbad, CA). Polyadenylated RNA was purified on oligo-dT columns from Stratagene (La Jolla, CA). Northern and Southern blotting were performed using standard techniques. Premade mouse tissue Northern blots purchased from OriGene Technologies (Rockland, MD) were hybridized and washed as recommended by the manufacturer.

DNA constructs

The construct pCI-HA3 was generated by cloning of annealed primers containing 2 copies of the HA epitope tag into Xhol-EcoRI sites of the mammalian expression vector pCI-neo (Promega, Madison, WI). The epitope-tagging at the amino-terminus of RP was performed through PCR amplification on a Marathon-Ready mouse 7-day embryo (Clontech, Palo Alto, CA) of the full-length rp cDNA, followed by in-frame cloning into EcoRI-Xbal sites of the pCI-HA3 (pCI-HA1-RP) vector. The insert of this plasmid was subcloned into Xhol-NorI sites of the vector pCDNA3.1 (Hygromycin (Invitrogen) to generate pCDNA-Hygro-HA1-RP. The full-length CDNA encoding wild-type RP was also subcloned in-frame into EcoRI-EcoRV sites of the expression vector pFLAG-CMV-6c (Sigma Chemical, St Louis, MO) and the HindIII site of the expression vector pHM6 (pHM6-RP, Roche, Indianapolis, IN).

Cells cultures and transfections

The generation of cell lines derived from mouse skin fibroblasts and culture conditions for these and human melanoma MNT-1 cells have been described previously.30 MNT-1 cells were stably transfected with pCDNA-Hygro-HA1-RP using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Hygromycin-resistant clones expressing HA1-RP were selected using 200 µg/mL Hygromycin B. Transient expression of HA1-RP and FLAG-RP was also performed using FuGENE 6 reagent.

Melanocyte lines melan-a39 and lines derived from rp/rp animals were cultured according to the protocols of Bennett et al39 (available online at http://www.sghms.ac.uk/depts/anatomy/pages/dcbandcm.htm). Mutant rp/rp melanocyte cell lines were generated by mating rp/rp mice with C57BL6/J mice carrying an Ink4a-Arf exon 2 deletion40 at Texas A&M University. Litters homozygous for both mutations were obtained at the third generation, and trunk skins from neonatal (aged 0-2 days) mice were sent in chilled culture medium to St George’s Hospital Medical School. There they were used as described previously for preparation of melanocyte cultures that were deficient in cell senescence to facilitate establishment as cell
lines.41 Three such lines were derived: melan-rp1, melan-rp2, and melan-rp3. In the studies described here, unless otherwise specified, melan-rp1 cells were used. Digital images were taken at room temperature using a Zeiss Axioplan 2 microscope equipped with an Axiocam high resolution camera (12 megapixels) and captured with Axiovision Imaging software v. 3.1 (all from Carl Zeiss, Welwyn Garden City, Great Britain).

Transfection of melan-a and melan-rp1 cultures with pHM6-RP was performed using GeneJuice transfection reagent (Novagen, Madison, WI).

Melanin assays and DOPA histochemistry
Melanin assays were based on the optical density at 475 nm (OD475) of cell lysates, as described.42 Melanin content was normalized to protein content, which was determined using a bichinchoninic acid assay (Pierce, Chester, United Kingdom).42 Dihydroxyphenylalanine (DOPA) histochemistry was carried out by a method modified from that described by Boissy et al.43 In brief, cells were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.2M sodium cacodylate buffer, pH 7.2, for 1 hour at room temperature. Cells were then washed 3 times for 5 minutes in 0.2 M sodium cacodylate buffer, pH 7.4, prior to incubation in l- or D-DOPA (0.1% in cacodylate buffer) for two 2.5-hour intervals at 37°C. (n-DOPA staining was used as a control and produced no stain.) Fresh DOPA solutions were used in the immunoprecipitation-recapture were performed as described.30

Immunoprecipitation-recapture assays
Immunoprecipitation-recapture assays for 1 hour at 37°C in the presence or absence of alkaline phosphatase (Roche), electrophoresed, and immunoblotted using anti-HA and anti-FLAG antibodies. In other experiments, HA-tagged RP was isolated from 32P-orthophosphate-labeled MNT-1 cells expressing HA3-RP by immunoprecipitation-recapture. The immunoprecipitates were washed twice with phosphorylation buffer consisting of 50 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8.5) and 0.1 mM EDTA and divided into 2 aliquots. One of the aliquots was treated with 0.2 U calf intestinal alkaline phosphatase (Roche) for 2 hours at 37°C; the other was mock-treated. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

Electrophoresis and immunoblotting
SDS-PAGE and immunoblotting analysis were performed as described.30 Horseradish peroxidase-labeled antibodies were detected by using the Western Lighting, Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA).

Electrophoresis and immunoblotting
SDS-PAGE and immunoblotting analysis were performed as described.30 Horseradish peroxidase-labeled antibodies were detected by using the Western Lighting, Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA).

Melanin assays and DOPA histochemistry
Melanin assays were based on the optical density at 475 nm (OD475) of cell lysates, as described.42 Melanin content was normalized to protein content, which was determined using a bichinchoninic acid assay (Pierce, Chester, United Kingdom).42 Dihydroxyphenylalanine (DOPA) histochemistry was carried out by a method modified from that described by Boissy et al.43 In brief, cells were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.2M sodium cacodylate buffer, pH 7.2, for 1 hour at room temperature. Cells were then washed 3 times for 5 minutes in 0.2 M sodium cacodylate buffer, pH 7.4, prior to incubation in l- or D-DOPA (0.1% in cacodylate buffer) for two 2.5-hour intervals at 37°C. (n-DOPA staining was used as a control and produced no stain.) Fresh DOPA solutions were used in the immunoprecipitation-recapture were performed as described.30

Immunoprecipitation-recapture assays
Immunoprecipitation-recapture assays for 1 hour at 37°C in the presence or absence of alkaline phosphatase (Roche), electrophoresed, and immunoblotted using anti-HA and anti-FLAG antibodies. In other experiments, HA-tagged RP was isolated from 32P-orthophosphate-labeled MNT-1 cells expressing HA3-RP by immunoprecipitation-recapture. The immunoprecipitates were washed twice with phosphorylation buffer consisting of 50 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8.5) and 0.1 mM EDTA and divided into 2 aliquots. One of the aliquots was treated with 0.2 U calf intestinal alkaline phosphatase (Roche) for 2 hours at 37°C; the other was mock-treated. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

Electrophoresis and immunoblotting
SDS-PAGE and immunoblotting analysis were performed as described.30 Horseradish peroxidase-labeled antibodies were detected by using the Western Lighting, Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA).

Immunofluorescence microscopy
Normal C57BL/6j and rpear melanocytes were plated on to coverslips in 6-well tissue culture plates at a density of 1 × 10^3 cells/well and allowed to grow for 48 hours. Then, 1 μg plasmid pHM6 or pHM6-RP per well was introduced into cells using GeneJuice transfection reagent (Novagen) according to the manufacturer’s protocol. Twenty-four hours after transfection, the cells were washed in phosphate-buffered saline (PBS), fixed in 1 mL 4% paraformaldehyde for 5 minutes, and washed 2 times with methanol. The cells were then fixed in methanol for an additional 10 minutes and washed 5 × 5 minutes in PBS. All washes and fixation steps were carried out on ice with ice-cold reagents. Subsequent steps were carried out at room temperature. Prior to the application of the primary antibody, coverslips were incubated for 30 minutes in 1 × PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100. Coverslips were inverted onto a 50-μL drop of PBS/BSA/Triton buffer containing 1.6 μg/mL affinity-purified rabbit antihemagglutinin (HA; Sigma Chemical) and incubated in a humidified chamber for 1 hour. Coverslips were washed with PBS, followed by three 5-minute incubations in PBS/BSA/Triton buffer. Prior to application to the fluorescently labeled secondary antibody at 5 μg/mL (goat anti–rabbit Alexa Fluor 488, Molecular Probes), nonspecific binding was blocked by incubating the cells in 5% normal goat serum (NGS; Jackson ImmunoResearch, West Grove, PA) in PBS for 30 minutes. Following incubation for 1 hour with secondary antibody, coverslips were washed and mounted on glass slides using SlowFade Antifade (Molecular Probes) according to the manufacturer’s instructions. Digital images were obtained at room temperature using a Leica DMRE microscope equipped with a Leica 40×/0.85 objective lens (Leica, Wetzlar, Germany). Images were captured with a Printronix Instruments (Princeton, NJ) camera model RTE/CCD 1300 Y/H using Basic Metamorph acquisition software (Universal Imaging, Downingtown, PA).

For direct staining of RP in melanocytes, a similar protocol was followed with these exceptions. Initial blocking of nonspecific binding was accomplished using a 1:3 dilution of BlokHen (Aves Labs), rinsed with 1 × PBS, and incubated with affinity-purified chicken anti-RP at 1.8 μg/mL. The secondary antibody used was Alexa Fluor 488-labeled goat anti–chicken (Molecular Probes) at 5 μg/mL.

Generation of double homozygotes
Mice doubly homozygous for rp and ruby-eye (ru) were generated by conventional interbreeding. The ruby genotype of F2 offspring was confirmed by PCR and restriction enzyme analysis. gDNA was amplified using rs-specific primers (forward 5’-GGT AAG GAC CAA CCA GAG CTT TCA AGC-3’, reverse 5’-CAA GTG TTC CCT TGT TGT GGA TTC AGG-3’), and the reaction product digested with BsrDI. A single 296-bp band is seen in ru/ru mice due to deletion of a BsrD1 site, whereas wild-type DNA gives fragments of 123 bp and 182 bp. Because these experiments were performed prior to the identification of the rp gene, F2 mice were genotyped for rp by crossing to rp/ru homozygotes. The
phenotypic distribution of the offspring was analyzed using a χ² test to determine the probable genotype. To be classified as an rp/rp homozygote, we observed at least 6 rp/rp pups (and no other phenotypes) from each animal (.25 > P > .01).

Screening of human HPS patients

PCR amplification products from fibroblast DNA were sequenced using a Beckman CEQ 2000 and the CEQ Dye Terminator Cycle Sequencing kit according to the manufacturer’s instructions.

Results

The rp mutation

The rp mutation (Figure 1A) was first reported in 1981 as a new coat color mutation with increased kidney lysosomal enzyme activity that mapped to mouse chromosome (Chr) 7. Since then rp has been classified as a mouse model of HPS. We have confirmed increased bleeding times in rp/rp mice (Table 1) and shown that the number of dense bodies is significantly decreased in rp/rp platelets compared to normal (Table 1; Figure 1B). Absent or decreased platelet dense bodies are considered the “gold standard” for a diagnosis of HPS. Consistent with decreased numbers of dense bodies and prolonged bleeding, homozygous rp platelet serotonin levels are significantly decreased (0.20 ± 0.05 μg/10⁹ platelets versus 5.37 ± 0.46 in wild type [X ± SE], n = 4, P < .001)]. All other hematologic parameters are normal (Table 1).

Melanosomes defects are evident in cultured rp/rp melanocytes. By light microscopy rp/rp cells appear pale compared to melan-a cells, suggesting an overall decrease in pigmentation (Figure 1C). Transmission electron microscopy confirms that rp/rp melanosomes are abnormal. All stages of melanosome development are found, as in wild type, but immature stages are more frequent, and some clustering of melanosomes is seen (Figure 1D). Melanin assays support the visual evidence. Melanin content was 143.82 ± 12.43 μg melanin/mg protein in rp/rp melanocytes versus 225.72 ± 12.96 μg melanin/mg protein in wild-type melanocytes (X ± SE, P < .02 in 3 assays from each of 3 cell lines of each genotype). Tyrosinase distribution within rp/rp melanocytes shown by L-DOPA staining is abnormal as well. Tyrosinase accumulates in the perinuclear region in rp/rp melanocytes (arrow) but is well distributed across the cell in wild-type melanocytes. Bar represents 0.5 μM. (E) Electron microscopy of L-DOPA–stained melanocytes. Mutant melanocytes show DOPA-positive tubular structures (arrow) not seen in wild-type cells. Bar represents 1 μM.

Positional cloning of rp

We fine mapped rp to a 0.5-cM interval between the Rtn2 (reticulon 2) gene and the anonymous sslp marker D7Mit57 on Chr 7 (Figure 2A). This interval encompasses 300 kb and contains more than 30 known or predicted transcripts (Celera Discovery System and Celera Genomics associated databases). No strong candidate genes

Table 1. Hematologic parameters in adult rp/rp mice

<table>
<thead>
<tr>
<th>GTP</th>
<th>WBC count, × 10⁹/L</th>
<th>RBC count, × 10¹²/L</th>
<th>HGB, g/L</th>
<th>HCT value</th>
<th>PLT count, × 10⁹/L</th>
<th>MPV, fL</th>
<th>BT, min.</th>
<th>DB*, no. per platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>10.7 ± 2.6 (5)</td>
<td>9.9 ± 0.1 (5)</td>
<td>149 ± 10 (5)</td>
<td>423 ± 0.009 (5)</td>
<td>0.99 ± 0.15 (5)</td>
<td>4.3 ± 0.3 (5)</td>
<td>1.3 ± 0.7 (6)</td>
<td>10.8 ± 4.0</td>
</tr>
<tr>
<td>rp/rp</td>
<td>8.6 ± 1.3 (7)</td>
<td>10.3 ± 0.7 (7)</td>
<td>151 ± 4 (7)</td>
<td>425 ± 0.010 (7)</td>
<td>1.05 ± 0.13 (7)</td>
<td>4.4 ± 0.3 (7)</td>
<td>13.5 ± 3.1 (6†)</td>
<td>0.8 ± 1.0†</td>
</tr>
</tbody>
</table>

All values are mean ± SD. Parenthetical values indicate the number of samples.

GTP indicates genotype; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; MPV, mean platelet volume; BT, bleeding time; DB, dense bodies.

†P < .001.

From www.bloodjournal.org by guest on August 18, 2017. For personal use only.
were contained within this interval. For that reason and because most HPS genes are novel, we chose to directly sequence the rp DNA compared to B6 DNA that creates a premature stop codon in one predicted coding sequence. \(BCO43666\) (Figure 2B). This substitution was subsequently confirmed in \(+/+\) and \(+/rp\) animals derived from the \(rp\) line by intercrossing obligate heterozygotes. The mutation removes an Fnu4HI site, changing 63- and 71-bp fragments to a 134-bp fragment. The polymorphism was used to demonstrate that the mutation segregates with the \(rp\) phenotype (Figure 2C). From this and other evidence described herein, we conclude that \(BCO43666\) is the \(rp\) gene (GenBank accession nos. AY515509 and BK005392).

**The \(rp\) gene structure, expression, and protein distribution**

The wild-type \(rp\) mRNA contains 1867 bp consisting of 2 exons, separated in the gDNA by a 694-bp intron. Exon 1 encodes 61 bp of 5' noncoding sequence. Exon 2 encodes 9 bp of 5' noncoding sequence, the entire coding sequence of 585 bp, and 1212 bp of 3' untranslated sequence. The gene encodes a 195-amino acid protein with a predicted molecular weight of 20.4 kDa and a pl of 4.84. There are no predicted transmembrane domains or coiled-coil domains. PROSCAN predicts a single protein kinase C phosphorylation site and several casein kinase II phosphorylation sites. Immunoblotting analysis using extracts of MNT-1 cells transiently expressing RP tagged with either a triple HA epitope (HA3-RP) or a FLAG epitope (FLAG-RP) shows the presence of 2 polypeptide bands with molecular masses of 30 kDa and 33 kDa for HA3-RP and 28 kDa for FLAG-RP, respectively (Figure 3A). Alkaline phosphatase treatment of the extracts increases the electrophoretic mobility of the 33 kDa (HA3-RP) and 30 kDa (FLAG-RP) bands, suggesting that these correspond to phosphorylated forms of RP (Figure 3A). The phosphorylation of RP was confirmed by metabolic labeling of HA3-RP-expressing MNT-1 cells with \(^{32}\)P-orthophosphate, followed by immunoprecipitation with antibodies to the myc (nonspecific control) or HA epitopes (Figure 3B). Together, these experiments reveal that HA3-RP is phosphorylated internally and that its specific control) or HA epitopes. The positions of molecular mass markers are indicated on the right. (B) The phosphorylation of RP was confirmed by metabolic labeling of HA3-RP-expressing MNT-1 cells with \(^{32}\)P-orthophosphate in the presence (+) or absence (−) of alkaline phosphatase, followed by immunoprecipitation-recapture with antibodies to the myc (nonspecific control) or HA epitopes. The positions of molecular mass markers are indicated on the right. (C) Comparison of human and mouse RP amino acid sequence reveals an overall identity of 87% (shaded residues). The C→T transition converts amino acid 80 (underlined, glutamine) to a stop codon. (D) OriGene Technologies Northern blot showing expression of \(rp\) in mouse tissues. The blot was hybridized with the same 672-bp fragment described for Figure 2C. Molecular weight marker positions are indicated on the left. (E) \(rp\) message levels appear normal in kidney (left) and cultured melanocyte (right) mRNA. Note that a lower amount of \(rp\) melanocyte mRNA was loaded, as judged by the intensity of the actin signal. Each lane contains \(2 \mu g\) mRNA. (F) Immunofluorescence image of B6 (F) and \(rp/rp\) (G) melanocytes transfected with pHM6-RP and stained with anti-HA. No staining was evident in melanocytes transfected with vector alone (not shown). (H) Merged Nomarski and stained (affinity-purified chicken anti-RP peptide antibody recognizing amino acids 1-12 of the RP protein) image showing that RP-containing vesicles (green structures) and melanosomes (dark structures) do not colocalize extensively. (I-J) B6 (I) and \(rp/rp\) (J) melanocytes stained with affinity-purified chicken anti-RP peptide antibody. No staining was seen when the primary antibody (affinity-purified chicken anti-RP) was eliminated (not shown). Original magnification \(<400\) for panels F-J.
specifically labeled with 32P and migrates as a 33-kDa species. The apparent molecular mass of the dephosphorylated band is larger than that predicted from its amino acid sequence, even accounting for the contribution of the triple-HA (24 kDa) or FLAG (21.5 kDa) epitopes, indicating that RP migrates anomalously on SDS-PAGE. No other motifs are present that might give clues to the function of RP. The wild-type mouse RP protein shows 87% identity with the human orthologue (GenBank accession no. BK005393; Figure 3C) and 94% with the rat (GenBank accession no. XM_218422; not shown). In rp/rp the premature stop codon occurs at nucleotide 308, truncating the protein at 79 amino acids (Figure 3C). Searches of BLASTn, BLASTp, and Swiss-Prot indicate the gene is confined to higher eukaryotes; no orthologues are seen in yeast, flies, or worms.

The rp gene is expressed ubiquitously (Figure 3D). In homozygous rp/rp tissues, there is no apparent loss of message, suggesting the mRNA is not subject to nonsense-mediated decay (Figure 3E). By immunofluorescence, the RP protein shows a punctate, cytoplasmic distribution in B6 melanocytes transfected with the normal RP cDNA in expression vector pHM6 (Figure 3F). The positive-staining structures extend well into the dendrites of the cells. A similar distribution is seen in transfected rp/rp melanocytes (Figure 3G). No staining was evident when cells were transfected with pHM6 vector alone (not shown). The RP-positive vesicles do not appear to colocalize with mature melanosomes (Figure 3H). Staining nontransfected cells using an affinity-purified peptide antibody raised in chickens that recognizes amino acids 1 to 12 of RP failed to label melanocytes (Figure 3I-J). No such signal is obtained in the absence of primary antibody. We presume that a truncated protein is being detected in rp/rp cells. However, definitive proof of this is not currently available because attempts to generate peptide antibodies to the C-terminus of RP have been unsuccessful. Further studies will be required to identify the precise structures containing RP. From the current analyses, we can only conclude that these structures appear normally distributed in rp/rp cells.

**RP assembles with components of BLOC-1 but not AP-3 or BLOC-3**

Loss of one BLOC-1 component has been shown to destabilize other subunits of the complex.21,28,51 Pallidin, a component of BLOC-1, is decreased in rp/rp fibroblasts compared to normal C57BL/6 (B6) fibroblasts (Figure 4A), suggesting that RP may be a BLOC-1 component. To examine this possibility directly, we stably transfected MNT-1 cells with HA3-RP and performed immunoprecipitation-recapture assays. Proteins were first immunoprecipitated with anti-HA, denatured, and then subjected to a second immunoprecipitation ("recapture")27 using antisera to the BLOC-1 components, pallidin, cappuccino, muted, and to the HA epitope. In each case, immunoprecipitation of HA3-RP with the BLOC-1 components was evident (Figure 4B). We also demonstrated coprecipitation by using antipallidin and anti-HA in the first and second rounds of immunoprecipitation, respectively (Figure 5). Furthermore, HA3-RP failed to coimmunoprecipitate with components of the AP-3 complex (rp3, m3) and BLOC-3 (HPS1, HPS4; Figure 5). The interaction of HA3-RP and pallidin was further examined by sedimentation velocity analysis on sucrose gradients using cytosol from MNT-1 cells stably expressing HA3-RP. Gradient fractions were assayed by immunoblotting with anti-HA (Figure 6). The distribution of HA3-RP on the gradient exhibited 2 peaks in fractions number 4 and 7-8, respectively (Figure 6A). Immunoprecipitation with antibodies against pallidin followed by immunoblotting with antibodies to HA (to reveal assembled HA3-RP) revealed a single peak (fraction 7) corresponding to a sedimentation coefficient of 5.2 S (Figure 6B). This peak coincided with the pallidin peak (Figure 6C). Therefore, the HA3-RP-pallidin complex sediments as a 5.2 S species, whereas the peak of HA3-RP in fraction 4 likely represents excess, unassembled HA3-RP. The sedimentation coefficient of the HA3-RP-pallidin complex was distinct from that of BLOC-3 as shown by immunoblotting with anti-HPS4 antibody (6.3 S, Figure 6D and Gautam et al29). Significantly, the electrophoretic mobility of the HA3-RP protein that cosediments with pallidin in fraction 7 (Figure 6E) is shifted upward. Similar results were obtained using Myc3-tagged RP (not shown). Together with the altered mobility on alkaline phosphatase treatment (Figure 3A) and 32P-incorporation studies (Figure 3B), these data indicate that the phosphorylated form of RP is a component of BLOC-1.

**Ruby-eye interacts semidominantly with rp**

Genetic complementation studies in mice frequently provide valuable insights into the functional interdependence of proteins or protein complexes. We produced mice doubly homozygous for rp and cno and rp and pc by conventional interbreeding for 2 generations. No exacerbation of the HPS phenotype was seen in either case (not shown). These results are consistent with the
independent pathways in the biogenesis of LROs. These data indicate that BLOC-1 and BLOC-2 define functionally mass markers are indicated on the right.

In Figure 4, we subjected the supernatant obtained after the recapture step (2nd step) to 2 additional IP steps (3rd IP and 4th IP) using antibodies to immunoprecipitation (1st IP), the Hermansky-Pudlak syndrome 1 (HPS1) protein, the Hermansky-Pudlak syndrome 4 (HPS4) protein, and mouse monoclonal anti-myc (control) or pallidin (PA), the Hermansky-Pudlak syndrome 1 (HPS1) protein, the Hermansky-Pudlak syndrome 4 (HPS4) protein, and mouse monoclonal anti-myc (control) or anti-HA were used in the 1st IP. Mouse monoclonal anti-HA was used in the 2nd IP step. To confirm the presence of AP-3, BLOC-1, and BLOC-3 complexes after the first immunoprecipitation (1st IP), the supernatant obtained after the recapture step (2nd IP) were subjected to 2 additional IP steps (3rd IP and 4th IP) using antibodies to α3 and α3, pallidin and cappuccino, or HPS1 and HPS4 or HPS4 and HPS1 in the consecutive IP steps (3rd IP and 4th IP), respectively. The positions of molecular mass markers are indicated on the right.

Biochemical data and provide further proof that RP is a component of BLOC-1. To test for an interaction of BLOC-1 and BLOC-2, we produced mice carrying various combinations of HPS5/ru and rp. Doubly homozygous offspring (rp/rp, ru/ru, and rp/rp, ru/ru) showed a exacerbated coat-color phenotype compared to either single homozygote alone (Figure 7). Notably, one copy of ru is sufficient to enhance the pigmentation defect in rp/rp mice; rp/rp, +/ru showed a phenotype intermediate to that of rp/rp, +/+ and rp/rp, ru/ru mice. These data indicate that BLOC-1 and BLOC-2 define functionally independent pathways in the biogenesis of LROs.

Human mutation screening

We screened for RP mutations 15 patients with HPS for which the genetic basis of their disease is unknown. No defects were detected.

Discussion

Increased bleeding times, coat color dilution, and elevated kidney lysosomal enzyme concentrations in rp/rp mice have been previously described.33,46 Here, we extend the observations to demonstrate a marked decrease in the number of platelet dense bodies, the hallmark of HPS, with concomitant decrease in platelet serotonin levels. In addition, a detailed analysis of cultured rp/rp melanocytes revealed abnormalities characteristic of HPS, specifically decreased numbers of melanosomes with decreased pigment content and increased numbers of small and immature melanosomes. In addition, tyrosinase trafficking is abnormal as evidenced by its accumulation in the perinuclear region and the presence of abnormal DOPA-positive tubular structures. Of note, rp/rp mice were originally reported to have normal melanosomes in the eye. However, a detailed electron microscopic analysis indicates this is not the case (B.G. and L.L.P., manuscript in preparation).

By fine-linkage mapping, we localized rp to a 300-kb interval on mouse Chr 7. Direct sequencing identified rp as a novel gene encoding a highly acidic (pI 4.84) protein that is strongly conserved with the human protein (87% identity). There are no predicted transmembrane domains, suggesting that the protein is cytosolic.
Wild-type RP protein localizes to punctate structures within the cytoplasm, reminiscent of other HPS proteins. In homozygous mutants, a premature stop codon is present at amino acid 80. However, mRNA levels are not affected, and the protein distribution appears normal at the light microscopy level, suggesting that the truncated protein is stable.

Loss of one subunit of a multiprotein complex frequently results in deficiency of other subunits. For example, loss of β3A destabilizes the AP-3 complex in HPS2/pearl, and loss of the CNO protein in cappuccino results in deficiencies of the BLOC-1 components pallidin and muted. In rp/rp fibroblasts, we observed that pallidin was decreased compared to wild-type fibroblasts, not only in deficiency of other subunits. For example, loss of dysbindin, a member of the biogenesis of lysosomal lineage: the Hermansky-Pudlak syndromes. Curr Mol Med. 2002;2:451-467.

Notably, the migration of the RP protein suggests that it is the phosphorylated form that specifically assembles within BLOC-1 complexes of BLOC-1 and BLOC-3, respectively, do not show an exacerbated phenotype, suggesting that BLOC-1 and BLOC-3 are functionally independent processes during the biogenesis of LROs. Interestingly, mice doubly homozygous for pallid and pale ear, components of BLOC-1 and BLOC-3, respectively, do not show an exacerbated phenotype, suggesting that BLOC-1 and BLOC-3 are functionally interdependent.

The mouse models of HPS have been invaluable in identifying gene defects in human HPS and in dissecting the protein building blocks participating in the fundamental processes of organelle biogenesis. Their participation in functionally distinct subcellular protein complexes is now well established. However, the precise interactions of most of the HPS proteins within these complexes and the interactions and functions of the complexes themselves remain to be established.

Note added in proof. While this manuscript was being submitted, Starcevic and Dell’Angelia identified the same mutation in rp/rp mice using a biochemical approach.

Acknowledgments

We thank Jane Barker and Linda Washburn for critical review of the manuscript.

References


Reduced pigmentation (rp), a mouse model of Hermansky-Pudlak syndrome, encodes a novel component of the BLOC-1 complex