The Hermansky-Pudlak Syndrome 3 (cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2)

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Running Title - Characterization of the BLOC-2 complex
SUMMARY

Hermansky Pudlak Syndrome (HPS) is a genetically heterogeneous inherited disease affecting vesicle trafficking among lysosome-related organelles. The Hps3, Hps5 and Hps6 genes are mutated in the cocoa, ruby-eye-2 and ruby-eye mouse pigment mutants respectively, and their human orthologs are mutated in HPS3, HPS5 and HPS6 patients. These three genes encode novel proteins of unknown function. The phenotypes of Hps5/Hps5,Hps6/Hps6 and Hps3/Hps3,Hps6/Hps6 double mutant mice mimic, in coat and eye colors, in melanosome ultrastructure and in levels of platelet dense granule serotonin the corresponding phenotypes of single mutants. These facts suggest that the proteins encoded by these genes act within the same pathway or protein complex \textit{in vivo} to regulate vesicle trafficking. Further, the Hps5 protein is destabilized within tissues of Hps3 and Hps6 mutants, as is the Hps6 protein within tissues of Hps3 and Hps5 mutants. Also, proteins encoded by these genes co-immunoprecipitate and occur in a complex of 350 kDa as determined by sucrose gradient and gel filtration analyses. Together, these results indicate that the Hps3, Hps5 and Hps6 proteins regulate vesicle trafficking to lysosome related organelles at the physiological level as components of the BLOC-2 (biogenesis of lysosome-related organelles complex-2) protein complex and suggest that the pathogenesis and future therapies of HPS3, HPS5 and HPS6 patients are likely to be similar. Interaction of the Hps5 and Hps6 proteins within
BLOC-2 is abolished by the 3 amino acid deletion in the \textit{Hps6}^\text{ru} mutant allele, indicating that these three amino acids are important for normal BLOC-2 complex formation.

\textbf{Keywords}: vesicle trafficking, Hermansky-Pudlak syndrome, lysosome-related organelles, pigment mutants

\section*{INTRODUCTION}

The cocoa (\textit{coa}/\textit{Hps3}), ruby-eye-2 (\textit{ru2}/\textit{Hps5}) and ruby-eye (\textit{ru}/\textit{Hps6}) mouse pigment genes encode novel proteins, which regulate the synthesis of lysosome-related organelles including melanosomes and platelet dense granules (1,2). \textit{Hps3}, \textit{Hps5} and \textit{Hps6} mutant mice have morphologically abnormal melanosomes and decreased quantities of intragranular components of platelet dense granules (3-6). Organellar trafficking abnormalities lead in turn to hypopigmentation of both coat and eyes and prolonged bleeding times. All three mutants are appropriate animal models for the inherited human disease Hermansky-Pudlak Syndrome (HPS) (MIM 203300) (7,8), which presents with similar abnormalities of subcellular organelles. Associated clinical symptoms of HPS include loss of visual acuity, prolonged bleeding and lung disease due to abnormalities of melanosomes, platelet dense granules and lysosomes respectively.

\textit{Hps3}, \textit{Hps5} and \textit{Hps6} mutant mice are among at least 16 mouse models of HPS (2,5). Human HPS patients with mutations in seven mouse HPS genes
have been identified (2,7,9). One class of five HPS genes (2) encodes proteins with established functions in vesicle trafficking to lysosome-related organelles in both lower and higher eukaryotes. In contrast, the second class of nine genes (2,10,11), which includes the Hps3, Hps5 and Hps6 genes of this report, are expressed only in higher eukaryotes and encode novel proteins with no recognizable structural motifs and whose functions are unknown. Most recently (9) the sandy (sdy/Hps7/Dtnbp1) gene was identified as encoding dysbindin, a dystrobrevin interacting protein (12).

The Hps5 and Hps6 proteins directly interact in a multiprotein complex termed biogenesis of lysosome organelles complex-2 (BLOC-2) (2). Hps3 mice have coat color (13) similar to that of Hps5 and Hps6 mutants which in turn are mimic mutants regarding coat and eye colors (2,14). These facts suggested that the function(s) of the Hps3, Hps5 and Hps6 genes are related and that they might be residents of a common protein complex. To test this hypothesis and to better understand the novel proteins of the BLOC-2 complex, we tested for epistatic interactions of the Hps3, Hps5 and Hps6 genes in doubly mutant mice and for complex formation by their protein products.

EXPERIMENTAL PROCEDURES

Mice

Mutant mice together with normal C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were subsequently bred and maintained in the animal facilities of Roswell Park Cancer Institute. Unless
indicated otherwise, the particular alleles utilized in these studies are as follows. The $Hps3^{coa}$ allele contains a splice site mutation resulting in a frameshift and loss of expression of the Hps3 mRNA (1); the $Hps5^{ru-2J}$ allele contains a frameshift mutation which causes loss of the C-terminal third of the Hps5 protein (2); the $Hps6^{ru}$ allele contains a small in-frame deletion which results in loss of 3 amino acids at positions 187-189 (2). The $Hps6^{ru-6J}$ mutation contains a 5.3 kb IAP element insertion which causes loss of transcript expression (2). The $Hps3^{coa}$ mutation arose and is maintained on the C57BL/10J background (15). Both the $Hps5^{ru-2J}$ and $Hps6^{ru}$ mutations arose on the C3H inbred strain background and were subsequently transferred to and maintained as congenic mutants on the C57BL/6J inbred strain background. All mice utilized in these experiments were 2-5 months old. All procedures (mouse protocol 125M) were reviewed and approved by the Roswell Park Institutional Animal Care and Use Committee and adhered to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Construction of double mutant mice**

Heterozygous F$_1$ offspring ($Hps3^{+}$/ and $Hps6^{+}$/ or $Hps5^{+}$/ and $Hps6^{+}$/) were produced by mating of $Hps3^{+}$/ and $Hps6^{+}$/ and $Hps5^{+}$/ and $Hps6^{+}$/ mice and were, as expected, of normal black coat and eye color. F$_1$ offspring were mated to produce an F$_2$ generation. F$_2$ mice doubly homozygous ($Hps3^{+}$/ and $Hps6^{+}$/ or $Hps5^{+}$/ and $Hps6^{+}$/) for mutant genes were verified by molecular diagnoses of genotype at each gene by PCR amplification.
and sequencing of normal and genomic tail DNA using appropriate primers (1,2) (sequences available upon request). Since double mutants appeared healthy and had no obvious reductions in breeding efficiency, they were mated among themselves to maintain the double mutant colonies. Since all single and double mutants are on the C57BL/6J strain background (or on the closely related C57BL/10J strain background in the case of the Hps3 mutant), contributions of background genes are essentially identical in all.

**Antibodies**

The peptide sequence, CNQERRGKPERIHVSSE, located near the amino-terminus of the Hps5 protein (2) was conjugated to carrier protein KLH, and a polyclonal antiserum was prepared in rabbits by Covance, Denver, PA.

To prepare antisera to the Hps6 protein, an expression plasmid pET 15b (Novagen) encoding the His-tagged C-terminal half (residues 1201 to 2418) of the Hps6 protein was transformed into E.coli BL21 DE3 (Novagen). The Hps6 protein was expressed within inclusion bodies, solubilized with 6M urea and purified by Ni$^{2+}$ sepharose affinity chromatography (16) followed by dialysis successively against 4M, 2M and no urea in 20 mM Tris-HCl buffer, pH 7.4. Rabbits were initially injected with 250 µg of purified His-Ruby protein followed by boosting with 125 µg biweekly before final collection of antisera at 100 days.

**Platelet collection and platelet serotonin analyses**

Platelets were harvested from the peripheral blood of normal and mutant mice in the presence of sodium citrate (17). Washed platelets were lysed in 1 ml
distilled water, counted in a Coulter Z2 particle count and size analyzer and assayed fluorometrically for serotonin (17).

**Immunoblotting**

Tissue extracts were subjected to denaturing SDS gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with nonfat dry milk or ECL Advance blocking agent in PBS with 0.1% Tween 20 for 1 hour followed by incubation with primary antiserum at 1:1000 dilution for 1 hour. After washing 1 hour with blocking solution the membrane was incubated with 1:50,000 dilution of anti Rabbit HRP-conjugated secondary antibody (Amersham Bioscience) for 1 hour and washed with PBS containing 0.1% Tween 20. Bound antibody was detected using the ECL Plus (+) system for Hps6 and ECL Advance for Hps5 (Amersham Pharmacia Biotech). Blots were calibrated with Kaleidoscope prestained molecular weight standards (Bio-Rad). Monoclonal mouse anti-α tubulin (Sigma) was used as loading control. Multiple exposures confirmed that blots were exposed within a linear range.

**Yeast Two-hybrid Analyses**

The Matchmaker GAL4 Two-Hybrid System 3 kit (Clontech) for two hybrid analyses was used at low and high stringency as described (Zhang et al., 2003). HPS6 mutant constructs in DNA binding domain (pGBKKT7) and activation domain (pGADT7) vectors were produced by deleting the 3 amino acids (His-Cys-Pro) at positions 187-189 from the wild type HPS6 cDNA. HPS6 alanine
mutant constructs were prepared by site directed mutagenesis by singly replacing each of these 3 amino acids in the wild type construct with alanine. All constructs were cloned in-frame to the DNA binding and activation domains of the Gal4 transcription factor and verified by sequencing. Plates were incubated at 30° C for 5 days and monitored for growth and blue color by visual inspection. To verify production of construct proteins in yeast, extracts from colonies growing on low stringency plates were immunoblotted with antibodies to either Myc (goat polyclonal, 1:250 dilution; Santa Cruz Biotechnology) or HA (mouse monoclonal antibody, 1:1000 dilution; Berkeley Antibody Company) epitopes, which were fused in-frame to cDNAs.

**Electron microscopy**

Eyes were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in spur resin as described (1) before viewing on a Siemens 101 Electron microscope at an accelerating voltage of 80 kV.

**Coimmunoprecipitation**

Open reading frames of *Hps3, Hps5, Hps6, Hps7, Ap3b1* and *pa* cDNAs were fused in-frame to pCMV-Tag vectors (Myc and Flag), and the resulting fusion constructs were verified by sequencing. Human embryonic kidney (HEK) 293 cells (3 x 10⁵) were cotransfected, using FUGENE6 (Roche), with epitope-tagged constructs at a ratio of 1:1, except in the cases of a) Hps5 Flag with Ap3b1-Myc, Hps6-Myc or Hps3-Myc (1.8:0.2), b) Hps7-Flag with coa-Myc
(0.5:1.5) and c) Hps6-Flag with Hps3-Myc (0.25:1.75). The cells were also singly transfected with Myc epitope tagged constructs of pa, Hps3, Hps5 and Hps6. At 48 h after transfection, proteins were solubilized with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors for 1 h at 4°C. Samples were immunoprecipitated by incubating them for 1 h at 4°C with Flag M2 antibody-conjugated agarose (Sigma), and washed with agarose beads three times with 0.5 M Tris-HCl, pH 7.4, plus 1.5 M NaCl. Bound proteins were eluted by treating the samples for 5 min at 95°C with denaturing Laemmli buffer, and blots of 8% SDS-PAGE gels were analyzed with either with rabbit polyclonal antibody against Flag (1:1,000 dilution; Affinity Bio-Reagents) or with goat polyclonal antibody against Myc (1:250 dilution; Santa Cruz Biotechnology). Horseradish peroxidase-linked donkey antibody against rabbit immunoglobulin G (1:5000 dilution; Amersham Life Sciences) was used as a secondary antibody for Flag blots, and horseradish peroxidase-linked bovine antibody against goat immunoglobulin G (1:5000 dilution; Santa Cruz Biotechnology) was used as a secondary antibody for Myc blots. Blots were treated with the enhanced chemiluminescence reagent (ECL +plus; Amersham, Piscataway, NJ) and exposed for 1 min.

Size-exclusion Chromatography and Sedimentation Velocity Analysis

Cytosolic extracts from the liver of normal and mutant mice were prepared by homogenization in Detergent-free Tris Buffer [0.3 M Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM MgCl₂, 1 mM 4-(2-aminoethyl)-
benzenesulfonyl fluoride, 10 mg/l leupeptin, 5 mg/l aprotinin and 1 mg/l pepstatin A], using a Dounce homogenizer, followed by centrifugation at 5000 X g for 5 min and then at 120,000 X g for 90 min, at 4°C. Size-exclusion chromatography was performed as described (18). Sedimentation velocity analysis was carried out by ultracentrifugation of cytosolic extract (0.2 ml, ~5 mg total protein) loaded on top of a linear 5-20% (w/v) sucrose gradient prepared in Detergent-free Tris Buffer (12 ml), for 13 h at 39,000 rpm on a SW41 rotor (Beckman Coulter). Fractions were collected from the bottom of the tube. Fractions resulting from both the size-exclusion chromatography and sedimentation velocity experiments were analyzed by immunoblotting using antibodies to Hps5 and Hps6 proteins. Cytosolic liver extracts from Hps5<sup>ru-2J</sup> and Hps6<sup>ru-6J</sup> null mutant mice were analyzed in parallel to confirm the identity of the Hps5 and Hps6 protein bands, respectively.
RESULTS

In vivo physiological interactions of Hps3, Hps5 and Hps6 proteins in the production of lysosome-related organelles in doubly mutant mice

To test for interactions between a) the Hps5 and Hps6 gene products and b) the Hps3 and Hps6 gene products at the physiological level, appropriate double mutant mice (i.e. homozygous for mutant genes at two of these HPS loci) were bred, verified (see Methods) and analyzed for abnormalities of melanosomes and platelet dense granules, the lysosome-related organelles most severely affected in Hps5 and Hps6 mutants.

The coat and eye colors of Hps5/Hps5, Hps6/Hps6 double mutants are hypopigmented in comparison to C57BL/6J controls and identical (Fig. 1a) to those of the Hps5/Hps5 and Hps6/Hps6 single mutants (which themselves exhibit [Fig 1a] mimic phenotypes) suggesting a common abnormality in melanosomes, the subcellular organelle which imparts coat and eye coloration. In both single and double mutants, coat colors are the classical (14) ruby color. Eye color in all is a light pink in offspring less than 1 week of age (not shown). This deepens in adults to a dark ruby eye color, distinguishable from the black eyes of normal C57BL/6J mice only when closely observed with intense light.

The detailed mimicry in pigmentation and melanosomal properties of the Hps5 and Hps6 single and double mutants extends to the ultrastructural level in melanosomes of the retinal pigment epithelia (RPE) and choroids of the eye (Fig. 2). Quantitatively, there are major and similar reductions in numbers of melanosomes of the RPE in single and double mutants. Likewise, all mutant
abnormalities are qualitatively concordant. The few remaining melanosomes within the RPE are often of unusual morphology, and some appear to be end-stage degradative organelles. All mutants contain larger numbers of choroidal than RPE melanosomes. However, all choroidal melanosomes are smaller than those of the control C57BL/6J and often have uneven “ragged” edges. The most conspicuous feature of the choroids of all single and double mutants is clumping (Fig. 2) of melanosomes within a single membrane-limited body, a morphological feature not observed in other mouse HPS mutants.

Identical conclusions apply to Hps3 and Hps6 single and double mutant mice, which were likewise bred and analyzed for abnormalities of coat and eye color and melanosome ultrastructure. Coat and eye colors of single (Hps3/Hps3 and Hps6/Hps6) mimic those of double (Hps3/Hps3,Hps6/Hps6) mutants (Fig. 1b). Likewise, the degree of hypopigmentation and the color of the eyes of newborn (not shown) Hps3/Hps3 and Hps3/Hps3,Hps6/Hps6 mutants mimic those of the above-described newborn Hps5/Hps5 and Hps6/Hps6 mice. At the ultrastructural level melanosomes of the RPE and choroid of double mutants are indistinguishable from those of all 3 single mutants and include the distinctive melanosome aggregates (Fig. 2) within the choroids. Taken together, these analyses of coat color and melanosome ultrastructure in three single and two double mutants suggest a common defect in the synthesis and/or processing of melanosomes in Hps3, Hps5 and Hps6 mutants.

Gene dosage does not affect the mutant phenotype since by visual examination, the Hps5/+,Hps6/Hps6; Hps5/Hps5, Hps6/+; Hps3/+,Hps6/Hps6
and $Hps3/Hps3,Hps6/+$ mice (not shown) are identical in coat and eye color to the original single mutant mice and to each other. Both single and double mutant mice appear healthy and robust for at least eight months of age.

A lysosome-related organelle invariably affected in all HPS patients and animal models is the platelet dense granule (5,7,8). Typically, platelet dense granules are either missing or greatly reduced in number. Alternatively, granules may be present, but are “empty”, a condition documented in $Hps3$, $Hps5$ and $Hps6$ mutants (15,19). Either condition leads to functionally abnormal platelets and prolonged bleeding times. The serotonin concentrations within platelet dense granules were indistinguishable among all single and double mutants, being greatly depressed to 6-8% that of normal C57BL/6J controls (Table 1). Consistent with these findings, all single and double mutants had bleeding times (not shown) greater than 15 min compared to the 2- 4 min times of C57BL/6J controls.

Combined, these several mimic effects on melanosomes and platelet dense granules suggest that the $Hps3$, $Hps5$ and $Hps6$ genes regulate the synthesis of lysosome-related organelles by a common mechanism at the physiological level.

**Test for destabilization of HPS5 and HPS6 proteins in extracts of other Hps mutants**

The above mimic effects of the $Hps3$, $Hps5$ and $Hps6$ genes suggested possible co-residence of their protein products within a common protein complex. An indication of residence of two proteins within a common protein complex is
destabilization of the partner protein within cells derived from mutants lacking one of the proteins, as loss of one member of a protein complex often leads to destabilization of other members of that complex (18,20). Accordingly, polyclonal antibodies to the Hps5 and Hps6 proteins were produced, and levels of these proteins were analyzed by western blotting in tissues of fourteen mouse HPS mutants and alleles to determine if mutations in other HPS genes affected their concentrations (Fig. 3). Consistent with its residence, together with the Hps6 protein, within the BLOC-2 complex (2), the Hps5 protein exhibits destabilization within spleen and lung extracts of the Hps6<sup>ru-6J</sup> null mutant (Fig. 3). Significant destabilization of Hps5 protein is also apparent in extracts of the Hps3 null mutant. Its concentration is also, as expected, depressed in extracts of single and double mutants containing the Hps5<sup>ru2-J</sup> allele, which have undetectable Hps5 protein levels, an expected result given the frameshift null mutation within this allele (2). Similar results were observed in heart extracts (not shown). No significant destabilization was apparent in any of the remaining 11 HPS mutants or the misty hypopigmentation mutant.

Similarly, in regard to possible residence within a common protein complex, there was a notable loss of Hps6 protein within brain and lung of the Hps3 (coa) and Hps5 mutants (Fig. 3). The latter result is consistent with evidence that both Hps5 and Hps6 proteins reside within the BLOC-2 complex (2). As expected, levels of the Hps6 protein were undetectable (Fig. 3) in extracts of the Hps6<sup>ru-6J</sup> allele, a null allele containing a 5.3 kb IAP element insertion within the Hps6 open reading frame (2). In contrast, there was substantial
expression of the Hps6 protein in mice carrying another Hps6 allele, Hps6\textsuperscript{ru}, which encodes a predicted relatively “mild” 3 amino acid deletion within the Hps6 protein (2). There were no losses of the Hps6 protein in brain of any of the other 11 mouse HPS mutants tested. Similar destabilizations of Hps6 protein were observed in spleen and heart (not shown). The destabilization of the Hps5 and Hps6 proteins in Hps3 tissues, together with prior evidence for complex formation between Hps5 and Hps6 proteins in BLOC-2 (2), suggested that all three proteins, Hps3, Hps5, and Hps6, reside within the BLOC-2 complex.

**Coimmunoprecipitation of the Hps3, Hps5 and Hps6 proteins**

To directly test for co-residence of the Hps3, Hps5 and Hps6 proteins within a common complex, epitope-tagged constructs of each gene were expressed within transfected cells, and immunoprecipitates were tested with appropriate antibodies (Fig. 4). Immunoblots of FLAG precipitates analyzed with the Myc antibody demonstrated that the Hps3, Hps5 and Hps6 proteins co-precipitated in all combinations, but did not interact with either the BLOC-1 proteins Hps7 (dysbindin) (9) or the Ap3b1 subunit of the AP-3 adaptor complex. As expected (9), the BLOC-1 components Hps7 and pallidin co-precipitated, but the BLOC-1 Hps7 protein did not precipitate the BLOC-2 component Hps3.

A second method of detection of interacting proteins, the yeast two-hybrid approach (Fig. 5) revealed the previously reported (2) interaction of the Hps5 and Hps6 proteins. There was, however, no direct interaction of the Hps3 protein with
either the Hps5 or Hps6 proteins (Fig. 5), suggesting that additional proteins bridging Hps3 with Hps5 and Hps6 are present in BLOC-2.

**Size-exclusion chromatography and Sedimentation Velocity Analyses**

The Hps5 and Hps6 proteins were found in common fractions in both size-exclusion chromatography (Fig. 6a) and sedimentation velocity (Fig. 6b) analyses of cytosolic liver extracts of C57BL/6J, consistent with coresidence within BLOC-2. The gel filtration results indicate that BLOC-2 has a Stoke's radius of 98 +/- 5 Angstroms, and the sedimentation coefficient, from the sucrose gradient is 8.3 +/- 0.5 S. It is an asymmetric complex with a frictional ratio (f/fo) of ~2. The calculated molecular mass of the complex is 350 kDa +/- 60 kDa.

**An Hps6 mutation (Hps6ru) abolishes interaction of the Hps5 and Hps6 proteins**

Previous studies (2) established that the Hps5 and Hps6 proteins directly interact in the formation of the BLOC-2 complex, as analyzed by both yeast two-hybrid and co-immunoprecipitation approaches. The mutation in the *ru* allele of the *Hps6* gene causes a limited molecular effect (deletion of the 3 amino acids, histidine-cysteine-proline, at positions 187-189 of the 805 aa protein). Nevertheless, this small alteration produces the abnormalities of lysosome-related organelles typical of the HPS phenotype. Therefore, we tested the hypothesis that this 3 amino acid region of the Hps6 protein is functionally important in interacting with the Hps5 protein. Consistent with this possibility, the
The *Hps6<sup>ru</sup>* allele causes no loss of expression of the Hps6 mRNA (2) and maintains significant expression of the Hps6 protein (Fig. 3). Indeed, yeast two-hybrid analyses (Fig. 5) indicated no interaction of the Hps5 and Hps6<sup>ru</sup> proteins. Interaction between the Hps5 and Hps6<sup>ru</sup> proteins was undetectable even after testing for long periods (7 days) of yeast growth. To determine if a particular residue among these three amino acids was critical for Hps5/Hps6 interaction, corresponding constructs in which only one of these three amino acids in the wild type Hps6 protein was substituted with alanine were tested. Each of these Hps6 mutant proteins (Fig. 5) retained full ability to interact with the Hps5 protein. Together these results suggest that the histidine-cysteine-proline motif indirectly mediates interaction between the Hps5 and Hps6 proteins. Loss of these 3 amino acids likely produces secondary structural alterations within the HPS6 protein to abolish its interaction with the HPS5 protein within BLOC-2.

**DISCUSSION**

Previous analyses by yeast two-hybrid and co-immunoprecipitation approaches (2) demonstrated that the Hps5 and Hps6 proteins interact to form the BLOC-2 complex. Our current data provides evidence that the Hps3 protein is likewise a member of this complex. All three (*Hps3, Hps5* and *Hps6*) single and two different double mouse mutants constructed from these single mutants are mimics in several vesicle-related phenotypes including eye and coat color, ultrastructural abnormalities of melanosomes of two tissues of the eye and diminution of platelet dense granule contents. Such genetic mimicry and absence
of epistatic interactions (21) strongly implies co-residence of affected proteins within a common vesicle trafficking pathway or a common protein complex. Destabilization of the Hps6 protein in both Hps5 and Hps3 tissues supports residence within a common protein complex, since cellular quality control mechanisms often lead to destabilization and degradation of incomplete protein complexes (18,20). Direct evidence for co-residence of the Hps3, Hps5 and Hps6 proteins within the BLOC-2 complex was provided by their coimmunoprecipitation from extracts of cells transfected with corresponding epitope-tagged constructs. The conclusion that the Hps3, Hps5 and Hps6 proteins are members of a common protein complex is likewise consistent with observed similarities in coat color intensity, as quantified by a Mexmeter, and in ultrastructure of cutaneous melanosomes of the Hps3, Hps5 and Hps6 mutants (22).

Deletion of the Histidine187-Cysteine-Proline189 motif in the Hps6ru allele causes the Hps6 phenotype by abolishing interaction of the Hps6 and Hps5 proteins within BLOC-2. The loss of interaction appears to occur by an indirect effect of the mutation on higher level folding since substitution of each of these three residues by alanine retained interaction. A practical consequence of these findings is that they strongly suggest that the Hps6ru allele is a functional null, a suggestion supported by the fact that the phenotypes of this allele and other null alleles at the Hps6 locus are indistinguishable.

Emerging themes from recent studies of HPS proteins are that a) they associate with other HPS proteins in complexes, and b) there are multiple complexes. The Ap3b1 and Ap3d proteins, mutated in the Hps2/pe (23,24) and
*mh* (25,26) mouse HPS mutants respectively are members of the AP-3 adaptor complex, which is well known to regulate trafficking of membrane proteins of vesicles in the trans-Golgi and endosomal compartments (27). The novel proteins encoded by the pallid, muted, cappuccino and *Hps7* (sandy) genes form the BLOC-1 complex (9,10,18,20) while the Hps1 and Hps4 proteins mutated in the *Hps1/ep* and *Hps4/le* mouse Hps mutants form the BLOC-3 complex (11,28,29). The recently identified buff HPS protein, encoded by the *Vps33a* gene (30) does not associate with known HPS proteins; however, like other HPS proteins, it mediates vesicle trafficking. In yeast it is, in combination with the Vps11, Vps16 and Vps18 proteins, a member of the class C vacuolar protein-sorting (*vps*) complex which mediates vesicle tethering and fusion with the yeast vacuole (31,32).

The molecular weight of the BLOC-2 complex, calculated from gel filtration and sedimentation velocity analyses is 350 +/- 60 kDa. This approximates the sum of the sequence-predicted molecular weights (327 kDa) of the mouse Hps3, Hps5 and Hps6 proteins, suggesting that BLOC-2 might be a heterotrimer containing one copy of each protein. However, the possible existence of additional subunits cannot be ruled out owing to the experimental error associated with this determination. Interestingly, yeast two-hybrid analyses (Ref 2 and this study) indicate that the Hps5 and Hps6 proteins directly interact while Hps3 is not directly bound to either. It is therefore possible that additional small proteins bridge between Hps3 and the other proteins of the BLOC-2 complex.
Similarly, unknown bridging proteins may exist in the BLOC-1 (18,20) and BLOC-3 (11,28,29) complexes.

Consistent with this study, the human HPS3, HPS5 and HPS6 proteins are likewise associated into a common complex (S. M. D. and E. C. D., unpublished observations). This finding in turn predicts similarity in phenotypes of HPS3, HPS5 and HPS6 patients. Indeed descriptions (2,33,34) of these patients indicate that all have a relatively mild form of HPS. HPS-3 manifests with mild oculocutaneous albinism, absent platelet dense granules and little or no pulmonary disease (34). HPS5 and HPS6 patients have mild oculocutaneous albinism together with prolonged bleeding (2). Consistent with the mild phenotypes of the human HPS3, HPS5 and HPS6 patients, the disease characteristics in Hps3, Hps5 and Hps6 mouse mutants are mild in comparison to all other mouse HPS mutants. Reductions in coat color in Hps3 and Hps5 mutants are relatively mild (22). Likewise, at the ultrastructural level, cutaneous melanosomes of Hps3, Hps5 and Hps6 mutants are only mildly affected, though there are significant increases in immature melanosomes (22). Our studies indicate significant abnormalities of melanosomes of the RPE and choroid in all BLOC-2 mutants. However, all phenotypes, including hypopigmentation, platelet functional abnormalities and lysosomal hyposcretion of BLOC-2 mutants are mild compared with members (pallid, muted, cappuccino and sandy) of another HPS protein complex, BLOC-1 (5,22,35).

There is presently no evidence that BLOC complexes directly interact. The present studies indicate that members of the BLOC-1 or AP-3 complex do not co-
precipitate with BLOC-2 subunits. Similarly, a wide variety of studies (2,9-11,18,20,28,29) have not detected physical interaction of BLOC-1, BLOC-2, BLOC3 or AP-3 complex components. Clearly, however, the related phenotypes of HPS patients and mouse mutants indicate that BLOC complexes must interact, at higher physiological levels, to regulate trafficking of lysosome-related organelles. There is in fact genetic evidence for physiological cooperation of BLOC-3 and the AP-3 complexes. Mice with mutations in both the AP-3 complex component Ap3b1 and the BLOC-3 component Hps1 exhibit a more severe phenotype than either single mutant (36). Based upon the prevalence of immature melanosomes in cutaneous melanocytes of Hps3, Hps5 and Hps6 mutants (1,22), it appears likely that BLOC-1 and BLOC-3 function at a similar very early stage of melanosome biogenesis while BLOC-2 and the AP-3 complex function at later stages.

With the exceptions of the AP-3 and class C protein complexes, there are limited clues to the molecular mechanisms by which the remaining HPS protein complexes regulate vesicle trafficking to lysosome-related organelles. All BLOC complexes are composed of novel proteins with no recognizable functional domains. It is likely that particular BLOCs interact with the actin cytoskeleton (18), control the subcellular localization of lysosomes (28) and serve at specific steps in melanosome biogenesis (22). In the mast cell, the HPS6 protein regulates the secretion of mast cell granules, which undergo unregulated kiss-and-run fusion with the plasma membrane in Hps6/Hps6 cells (37).
The findings that the Hps3, Hps5 and Hps6 proteins are members of a common BLOC-2 complex provide potentially useful information for future therapeutic interventions. A therapy stabilizing the BLOC-2 complex or correcting abnormal BLOC-2 function in any one of the HPS-3, HPS-5 or HPS-6 syndromes may likewise be applicable to the remaining two.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grants HL51480, HL31698 and EY12104 (R. T. S.) and HL68117 (E. C. D.). This research utilized core facilities supported in part by RPCI’s NCI-funded Cancer Center Support Grant, CA-16056. We thank Donna Reddington, Debra Tabaczynski and Mary Kay Ellsworth for excellent technical assistance.
FIGURE LEGENDS

Fig. 1. Mimicry of coat and eye hypo-pigmentation in (A) single and double \textit{Hps5} and \textit{Hps6} mutants and (B) single and double \textit{Hps3} and \textit{Hps6} mutants.

Fig. 2. Identical ultrastructure of melanosomes of the retinal pigment epithelium (RPE) and choroid of single and double mutant mice. The interface between the RPE (above) and choroid (below) regions is indicated by the hyphenated partial line at left. Arrows indicate clumps of melanosomes within a single membrane-limited body. Scale bars, 2 µm.

Fig. 3. Expression of \textit{Hps5} (ru2) and \textit{Hps6} (ru) proteins in extracts of various HPS mutants. A. Spleen extracts (20 µg protein) were blotted and incubated with rabbit anti-\textit{Hps5} serum (above) and later with monoclonal anti-\textit{α}-tubulin as a loading control. Brain extracts (20 µg) were similarly analyzed (below) with anti-\textit{Hps6} serum. Extracts of \textit{Hps5}/\textit{Hps5} (lane 4) and \textit{Hps5}/\textit{Hps},\textit{Hps6}/\textit{Hps6} (lane 5) mutants served as null controls for expression of the \textit{Hps5} protein while extracts of \textit{Hps6}^{ru-6J}/\textit{Hps6}^{ru-6J} mutants (lane 3) served as a null control (2) for expression of the \textit{Hps6} protein. It is known that these mutants produce little (\textit{gm/gm} and \textit{Hps6}^{ru}/\textit{Hps6}^{ru}) or none (\textit{Hps1}/\textit{Hps1}, \textit{Ap3b1}/\textit{Ap3b1}, \textit{Hps3}/\textit{Hps3}, \textit{Hps4}/\textit{Hps4}, \textit{Hps5}/\textit{Hps5}, \textit{Hps6}^{ru-6J}/\textit{Hps6}^{ru-6J}, \textit{Hps7}/\textit{Hps7}, \textit{Rab27a}/\textit{Rab27a}, \textit{mu/mu}, \textit{pa/pa},) or an unknown (\textit{rp/rp}, \textit{sut/sut}, \textit{Vps33a}/\textit{Vps33a}, and \textit{m/m}) quantity of the proteins encoded by their respective genes. C57BL/6J serves as an appropriate control strain for most of the mutants except for \textit{mu/mu} (\textit{mu/+}), \textit{sut/sut}, \textit{Hps6}^{ru-6J},
Rab27a/Rab27a (C3H/HeSnJ) and Hps7/Hps7 (DBA/2J). While misty (m/m) mutants do not fit the strict definition of HPS, they do have alterations of melanosomes and decreased ADP in platelet dense granules (38). Spleen extracts were utilized for Hps5 protein expression as it is expressed at very low levels in brain.

**B.** Lung extracts (20 µg protein) of Hps3, Hps5 and Hps6 mutants were analyzed with Hps5 (above) and Hps6 (below) antiserum.

**Fig. 4. The Hps3, Hps5 and Hps6 proteins co-immunoprecipitate.** HEK-293 cells were co-transfected with epitope-tagged constructs expressing the indicated proteins and analysed by immunoblotting 48 h after transfection. Proteins in c and d were immunoprecipitated with flag M2 antibody-conjugated agarose. a, Flag input in total lysates (6% of the total lysate) detected by polyclonal antibody against Flag. b, Myc input in total lysates (18% of the total lysate) detected by polyclonal antibody against Myc. c, Immunoprecipitated Flag protein (18% of lysate) detected by polyclonal antibody against Flag. d, Specific interactions (18% of the lysate) detected in the Flag immunoprecipitate by antibody against Myc.

Mobility positions of individual proteins are shown at left. Western blot analyses of extracts of cells singly transfected with Myc tagged constructs of pa, Hps3, Hps5 and Hps6 immunoprecipitated with Flag antisera were negative when analyzed with the Myc antibody (not shown). The Hps3-Flag construct was not analyzed since it failed to express in HEK-293 cells.
Fig. 5. Deletion of 3 amino acids in the *Hps6*<sup>ru</sup> allele abrogates interaction of the HPS5 and Hps6 proteins. Yeast strain AH109 was transformed with constructs expressing the indicated proteins fused to binding domains (left) or activation domains (top). We spotted co-transformants on plates containing high stringency (top) and low stringency (bottom) medium and assessed interaction of proteins by growth and blue color on high stringency plates. Co-transformants pGBK7-T53 and pGADT7-T are a positive control for interacting proteins; pGBK7-LAM and pGADT7-T are a negative control. The protein product of the *Hps6*<sup>ru</sup> allele lacks amino acids 187-189 (His-Cys-Pro). Each of these three amino acids of the wild type Hps6 protein is singly replaced by alanine in the Hps6Ala187, Hps6Ala188 and Hps6Ala189 constructs respectively. Protein expression in yeast cells in all cases of negative interactions was confirmed by immuno-blotting (not shown).

Fig. 6. Estimation of the size of BLOC-2 under non-denaturing conditions. (A) Size-exclusion chromatography. Liver cytosol from C57BL/6J mice was fractionated on a Superose 6 column as described (18). Fractions were analyzed for the presence of Hps5 and Hps6 proteins by immunoblotting. The exclusion volume (Vo) and the elution positions of standard proteins (Stokes radii given in Ångstroms) are indicated on the top. Fractions 22, 41, 43, 45 and 47 are not shown. (B) Sedimentation velocity analysis. Liver cytosol from C57BL/6J mice was fractionated by centrifugation on a 5-20% (w/v) sucrose gradient, as described under "Experimental Procedures." Fractions were analyzed for the presence of Hps5 and Hps6 proteins by immunoblotting. Fractions 1 (not shown) and 28 correspond to the top and bottom of the gradient,
respectively. The position of standard proteins (sedimentation coefficients given in Svedberg units) are indicated on the top. Not shown are fractions 1, 19, 21, 23, 25 and 27.

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Fig. 4

- **a)** Blot: Anti-Flag (Input)
- **b)** Blot: Anti-Myc (Input)
- **c)** IP: Anti-Flag
  Blot: Anti-Flag
- **d)** IP: Anti-Flag
  Blot: Anti-Myc

- **Hps5**
- **Hps6**
- **Hps7**
- **Hps5, Ap3b1**
- **Hps3**
- **Hps6**
- **pa**
Fig. 6

A. Gel Filtration

B. Sucrose Gradient
The Hermansky-Pudlak syndrome 3 (cocoa) protein is a component of the biogenesis of lysosome-related organelles complex-2 (BLOC-2)

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J. Biol. Chem. published online January 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311311200

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