Identification of Snapin and Three Novel Proteins (BLOS1, BLOS2, and BLOS3/Reduced Pigmentation) as Subunits of Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1)*

Received for publication, March 5, 2004, and in revised form, April 20, 2004
Published, JBC Papers in Press, April 21, 2004, DOI 10.1074/jbc.M402513200

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Biogenesis of lysosome-related organelles complex-1 (BLOC-1) is a ubiquitously expressed multisubunit protein complex required for the normal biogenesis of specialized organelles of the endosomal-lysosomal system, such as melanosomes and platelet dense granules. The complex is known to contain the coiled-coil-forming proteins, Pallidin, Muted, Cappuccino, and Dysbindin. The genes encoding these proteins are defective in inbred mouse strains that serve as models of Hermansky-Pudlak syndrome (HPS), a genetic disorder characterized by hypopigmentation and platelet storage pool deficiency. In addition, mutation of human Dysbindin causes HPS type 7. Here, we report the identification of another four subunits of the complex. One is Snapin, a coiled-coil-forming protein previously characterized as a binding partner of synaptosomal-associated proteins 25 and 23 and implicated in the regulation of membrane fusion events. The other three are previously uncharacterized proteins, which we named BLOC subunits 1, 2, and 3 (BLOS1, -2, and -3). Using specific antibodies to detect endogenous proteins from human and mouse cells, we found that Snapin, BLOS1, BLOS2, and BLOS3 co-immunoprecipitate, and co-fractionate upon size exclusion chromatography, with previously known BLOC-1 subunits. Furthermore, steady-state levels of the four proteins are significantly reduced in cells from pallid mice, which carry a mutation in Pallidin and display secondary loss of other BLOC-1 subunits. Yeast two-hybrid analyses suggest a network of binary interactions involving all of the previously known and newly identified subunits. Interestingly, the HPS mouse model strain, reduced pigmentation, carries a nonsense mutation in the gene encoding BLOS3. As judged from size exclusion chromatographic analyses, the reduced pigmentation mutation affects BLOC-1 assembly less severely than the pallid mutation. Mutations in the human genes encoding Snapin and the BLOS proteins could underlie novel forms of HPS.

The biogenesis of specialized organelles of the endosomal-lysosomal system, such as melanosomes and platelet dense granules, is controlled by a number of genes that are defective in various forms of Hermansky-Pudlak syndrome (HPS)1 in humans and inbred mouse models of the disease (1–3). Consistent with the roles of melanosomes in melanin synthesis and storage and of platelet dense granules in activation of platelet aggregation, the clinical manifestations common to all forms of HPS are hypopigmentation of hair, skin, and eyes and extended bleeding times due to storage pool deficiency (1, 2). Both melanosomes and platelet dense granules are referred to as “lysosome-related organelles” based on genetic and morphological evidence that supports the idea of a common biogenesis pathway with lysosomes (4–6). Other lysosome-related organelles include azurophil granules (in neutrophils), lytic granules (in cytotoxic T lymphocytes and natural killer cells) and lamellar bodies (in type II lung epithelial cells). Interestingly, additional manifestations observed in subsets of HPS patients and mouse HPS models can be ascribed to defects in lysosomes or related organelles other than melanosomes and platelet dense granules. These manifestations include defective lysosomal enzyme secretion reported for kidney and thrombin-stimulated platelets of several HPS mouse models (reviewed in Ref. 7), pulmonary fibrosis (probably due to abnormal lamellar bodies; see Refs. 8 and 9), impaired T-cell-mediated killing activity due to defects in lytic granules (10), and mild cedoid lipofuscinosis (presumably due to suboptimal lysosomal degradation; reviewed in Ref. 1). The fact that all genes currently implicated in the pathogenesis of HPS are expressed in a wide variety of cell types (11–16) also supports the notion that their function may be more general than regulating the biogenesis of only melanosomes and platelet dense granules.

Few of the genes known to be defective in HPS in humans and/or HPS-like phenotype in mice have been functionally characterized. Examples include those encoding the β3A and δ subunits of the AP-3 complex, which recognizes sorting signals of integral membrane proteins for transport to lysosomes and related organelles (17), and the gene encoding Vps33a, an ortholog of the yeast Vps33 protein implicated in membrane fusion events within the endosomal-lysosomal system (18, 19). On the other hand, most genes known to be associated with HPS encode polypeptides that display no homology to any characterized protein. Biochemical analyses of these polypeptides revealed that they are associated into at least three distinct multisubunit complexes, named BLOC-1, -2, and -3. Thus, BLOC-1 is known to contain Pallidin, Muted, Cappuccino, and Dysbindin (16, 20–22), BLOC-2 contains HPS3, HPS5, and HPS6 (15, 23, 24), and BLOC-3 contains HPS1 and HPS4.

* This work was supported in part by National Institutes of Health Grant HL68117. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY531265 and AY531266.

‡ Supported by United States Public Health Service National Research Service Award GM07104.
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1 The abbreviations used are: HPS, Hermansky-Pudlak syndrome; 3AT, 3-amino-1,2,4-triazole; BLOC, biogenesis of lysosome-related organelles complex; BLOS, BLOC subunit; GST, glutathione S-transferase; ORF, open reading frame; RT, reverse transcriptase.
(25–27). The possible existence of BLOC-4 and BLOC-5 complexes, sharing some BLOC-3 subunits, has been suggested by one group (25).

The work described herein addresses BLOC-1. The genes encoding the Pallidin, Muted, Cappuccino, and Dystin subunits are mutated in the pallid, muted, cappuccino, and sandy mouse strains, respectively, which display the most severe coat color dilution phenotypes among known mouse mutants. For BLOC-4, a nonsense mutation in human Dystin has been implicated in the pathogenesis of HPS type 7 (16). Biochemical studies have shown that the complex exists in soluble (cytosolic) and peripheral membrane protein pools (20, 21). On the basis of its hydrodynamic properties, the soluble form of bovine and mouse BLOC-1 was predicted to be asymmetric (frictional ratio >2) and to have a native molecular mass of about 200 kDa (16, 20). Importantly, immunoprecipitation of the complex under nondenaturing conditions suggested the possible existence of additional subunits (20, 21). Whereas the molecular function of BLOC-1 remains unknown, some clues have stemmed from the description of potential binding partners of known function. These binding partners include Syntaxin 13 (a SNARE family member involved in membrane fusion at early stages of the endocytic pathway), the α- and β-dystrobrevins (two dystrophin-related proteins that, like Dystrophin, are components of large membrane-associated complexes implicated in membrane stability and signaling events), and actin filaments (20, 28, 30).

In this paper, we report identification of four previously unknown subunits of BLOC-1. One of them is Snapin, a coiled-coil-forming protein originally described as a binding partner of SNAP-25 and SNAP-23, two closely related SNARE proteins implicated in membrane fusion events (31, 32). The other three are novel proteins herein referred to as BLOC subunits 1, 2 and 3 (BLOS1, BLOS2, and BLOS3). We also provide evidence that the mouse gene encoding BLOS3 is mutated in the HPS model strain, reduced pigmentation (AH109, as described previously (20)).

**Identification of Previously Unknown Subunits of BLOC-1**

Partially purified BLOC-1 was fractionated by gel filtration on a Superose 6 column (2.6 × 30 cm) equilibrated in Buffer B. Elution was performed in the same buffer at a flow rate of 2 mL/minute. The eluting material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with antibodies to Pallidin, Muted, or Dystin, and with polyclonal antibodies raised against recombinant GST-Dysbindin224 (GenBank™ NM_000195), were cloned into the EcoRI-SalI sites of the pGBT9 vectors (Clontech) to obtain in-frame fusions with Gal4 DNA-binding domains, and those containing the Pallidin subunit of BLOC-1, and those containing GST-Dysbindin224 were eluted in 0.1 M triethanolamine (pH 11.5) and collected into a tube containing the protease inhibitor mixture. The resuspended material was cleared by centrifugation at 120,000 × g for 30 min and loaded onto a Superose 6 column (2.6 × 32 cm) connected to a Fast Protein Liquid Chromatography system (Amersham Biosciences) and equilibrated in Buffer E. Elution was performed in the same buffer at a flow rate of 2 mL/minute. Collected fractions (4 mL) were analyzed by immunoblotting for the presence of the Pallidin subunit of BLOC-1, and those containing significant amounts of the complex (fractions 23–27) were pooled. The pooled sample was cleared by passage through a column containing Protein A-Sepharose fast flow beads (Amersham Biosciences) and then applied to an immunosorbent column that was prepared by cross-linking Protein A-Sepharose fast flow beads (0.7 mL) using 40 mM dimethylpimelimidate in 0.1 M sodium borate (pH 8.0). Subsequently, the column was washed with 70 volumes of Buffer B. Purification of BLOC-1 was monitored by SDS-PAGE of the bead-buffered supernatant and 0.1% (w/v) Triton X-100, 5 mM EDTA, 0.02% (w/v) sodium azide) followed by 15 column volumes of Buffer C containing 0.01% (w/v) SDS was then added. After incubation, the column volumes of the bead-buffered supernatant were eluted in 0.1 M triethanolamine (pH 11.5) and collected into a tube containing 1 mL of Buffer C (pH 7.5). Eluted proteins were concentrated by chloroform/methanol precipitation (34).

**Recombinant Protein Expression and Purification—**Plasmids encoding GST- and histidine-tagged proteins were transformed into E. coli DH5α (Invitrogen) and BL21-CodonPlus-(DE3)-RP (Stratagene), respectively. GST fusion proteins were affinity-purified on conjugated GST-Sepharose 4 Fast Flow beads (Amersham Biosciences). Recombinant histidine-tagged proteins were purified on TALON® Superflow resin (Clontech). Purified recombinant proteins were dialyzed against 50 mM Hepes buffer (pH 8.0).

**Antibodies—**Polyclonal antibodies to Pallidin and Muted were described previously (20). Polyclonal antibodies against Dystin, BLOS2, and BLOS3 were raised by immunizing rabbits with purified GST-Dysbindin224, GST-BLOS2, and GST-BLOS3, respectively, and then affinity-purified using the corresponding histidine-tagged segments of the immunogens covalently coupled to Affi-Gel 15 beads (Bio-Rad). Antibodies to Snapin and BLOS1 were generated by immunizing mice with GST-Snapin and GST-BLOS1, respectively, and then affinity-purified using immobilized GST protein. Anti-α-tubulin monoclonal antibody was purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences.

**Experimental Procedures**

**DNA Constructs—**Yeast two-hybrid plasmids encoding human Pallidin or Muted proteins fused in frame to Gal4 DNA-binding or activation domains have been described previously (20). The cDNA segments comprising the complete ORFs of human Dysbindin and Pallidin were amplified from the pGADT7 vector (Clontech) using a MATCHMAKER cDNA library (in the pGADT7 vector) derived from HeLa cells (Clontech). The resulting plasmid clones were selected on SD minimal medium (0.7% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, pH 5.5) supplemented with appropriate auxotrophic requirements except for leucine, tryptophan, and histidine and containing 5 mM 3AT. Colonies that developed within 3 days of culture at 30 °C were transferred to fresh SD medium supplemented as above or modified to contain 10 mM 3AT or to lack 3AT and adenine. Colonies that were able to grow on all of these media were selected, and the cDNA inserts in the activation domain plasmid were PCR-amplified and sequenced.

Yeast two-hybrid assays aimed at testing specific interaction pairs were carried out by double transformation of haploid S. cerevisiae strain AH109, as described previously (20).
MA) for in-gel tryptic digestion followed by microcapillary reverse-phase high performance liquid chromatography and nanoelectrospray tandem mass spectrometry on a Finnigan CLQ DECA XP Plus quadrupole ion trap mass spectrometer. Proteins in the gel sample were identified by correlating the tandem mass spectrometry spectra with known sequences in public protein data bases, using the Sequest algorithm (36) and programs developed in the Harvard Microchemistry Facility (37).

Other Biochemical Procedures—Protein concentration was estimated by Bradford's method using a commercial reagent (Bio-Rad) and bovine serum albumin as the standard. Immunoblotting and immunoprecipitation-recapture were performed as described (20, 38). Liver cytosolic extracts from the mouse strains C57BL/6J (wild type), pallid (B6.Cg-Pldnpa/J, formerly C57BL/Aj-pe), and reduced pigmentation (C57BL-rp/J, purchased from Jackson Laboratories) were prepared by homogenization of minced tissue in Buffer B with protease inhibitors followed by centrifugation at 5,000 \( \times g \) for 5 min and at 120,000 \( \times g \) for 90 min at 4 °C. For size exclusion chromatography, liver cytosol (0.2 ml, ~6 mg of total protein) was applied to a Superox 6 column (1 × 60 cm) equilibrated and eluted at 4 °C with Buffer B with protease inhibitors at a flow rate of 0.4 ml/min. Fractions (0.4 ml) were collected and analyzed by immunoblotting.

The column was calibrated using blue dextran (to determine exclusion volume) and the following standard proteins purchased from Sigma (Stokes radii in parentheses): bovine thyroglobulin (85 Å), bovine serum albumin (36 Å), carbonic anhydrase (24 Å), and horse heart cytochrome c (17 Å).

**Mutation Analysis and Real-time PCR**—Total RNA was isolated from liver and cultured skin fibroblasts (26) using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The ORF of BLOS3 was amplified by RT-PCR using the TITANiUM™ one-step RT-PCR kit (BD Biosciences). The RT-PCR product was subsequently gel-purified and sequenced at the UCLA Sequencing and Genotyping Core. For real-time PCR analysis, specific primer sets were designed to amplify a fragment of BLOS3 and of glyceraldehyde-3-phosphate dehydrogenase (primer sequences are available upon request). Total RNA isolated from fibroblasts was treated with the TURBO DNA-free™ kit (Ambion) to minimize contamination by genomic DNA and subsequently subjected to reverse transcription using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Real-time PCR was performed on an iCycler™ Thermal Cycler (Bio-Rad) using the iQ™ SYBR® Green Supermix (Bio-Rad). Data were analyzed using the MyiQ software package (Bio-Rad). The signal obtained for BLOS3 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (as a housekeeping control).

**RESULTS**

**Identification of Candidate Subunits of BLOC-1**—In order to identify previously unknown subunits of BLOC-1, both biochemical and yeast two-hybrid approaches were undertaken.

The biochemical approach involved purifying BLOC-1 by column chromatography and identifying candidate components by mass spectrometry. Bovine liver cytosol was chosen as the starting material, in part due to the availability of large quantities of tissue from a commercial vendor. By quantitative immunoblotting using our antibody to Pallidin, we estimated that the complex may need to be enriched up to 18,000 times to purify BLOC-1 by additional conventional or high performance chromatographic steps (e.g. strong anion exchange, hydrophobic interaction, hydroxyapatite) were hampered by aggregation of the complex under a wide variety of experimental conditions (data not shown). Consequently, the pooled sample resulting from the third purification step (i.e. size exclusion chromatography) was chosen at the starting material for immunooaffinity chromatography on an anti-Pallidin column, as described under “Experimental Procedures.” Although low protein concentration of the purified eluate precluded the quantification of enrichment and yield, we observed a dramatic difference in the protein band patterns, as visualized by SDS-PAGE, of the sample loaded on the column and the eluate (Fig. 1C). In addition, the apparent enrichment in Pallidin correlated with that in Dysbindin (Fig. 1D), suggesting that the entire complex, and not just Pallidin, was purified by this step.
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To identify proteins that were present in the partially purified BLOC-1 preparation, the sample was resolved by SDS-PAGE, and a portion of the gel containing protein bands of apparent molecular masses in the 13-34-kDa range (Fig. 1C, asterisk) and a second portion containing high molecular mass proteins (not shown) were excised and submitted for proteolytic digestion and peptide identification by tandem mass spectrometry. Whereas no known or candidate BLOC-1 subunits could be unambiguously identified in the high molecular mass region of the gel (data not shown), several were detected in the low molecular mass region. Specifically, peptides covering 26, 5, and 3% of the predicted amino acid sequences of the known BLOC-1 subunits Pallidin, Muted, and Dysbindin, respectively, were detected in the sample (Table I). Whereas Pallidin and Muted (apparent molecular masses 23–25 kDa) (20, 21) were expected to be present in the low molecular mass region and may correspond to one of the major protein bands of the sample (Fig. 1C), the presence of peptides derived from Dysbindin was not expected and may have resulted from partial degradation during purification. The proteins identified as candidate BLOC-1 subunits were Snapin (31) and three novel proteins that we named BLOS1, BLOS2, and BLOS3. The peptides detected accounted for 38, 72, 26, and 13% of the predicted amino acid sequences of Snapin, BLOS1, BLOS2, and BLOS3, respectively. Most of these proteins were also found in a second BLOC-1 purification experiment (Table I). Additional proteins deemed to represent contaminants were also detected (e.g. bovine immunoglobulin light chain, actin, and formyltetrahydrofolate dehydrogenase; data not shown).

The deduced amino acid sequences of Snapin, BLOS1, and BLOS2, but not BLOS3, contain regions predicted to form coiled-coil structures (data not shown). Like the previously known BLOC-1 subunits, these proteins are relatively small (<40 kDa) and have no recognizable homologues in yeast. Although BLOS1 is identical to a hypothetical protein named GCN5L1 (general control of amino acid synthesis 5-like 1) on the basis of a proposed sequence homology with the yeast GCN5 protein (39), data base searches using BLASTP and PSI-BLAST programs as well as pairwise sequence comparisons revealed that the proposed sequence homology is statistically not significant. In contrast, similar data base searches identified histone acetylase PCAF (p300/CREB-binding protein-associated factor; SwissProt accession number Q92831) as the most likely mammalian counterpart of yeast GCN5 (E value = 4 × 10⁻⁸⁰). Therefore, we will hereafter refer to GCN5L1 (also called RT14) (40) as BLOS1.

As a complement to the biochemical approach, we performed a yeast two-hybrid screen of a HeLa cDNA library, using full-length human Pallidin as the bait. Screening of 1.8 × 10⁷ cells containing both bait and prey plasmids yielded 224 colonies that were able to grow in the absence of histidine and in the presence of 5 mM 3AT (which inhibits the growth of cells expressing low levels of the His3 reporter gene; see Ref. 41). Based on the expectation that the interaction between Pallidin and other BLOC-1 subunits would be robust in the context of the yeast two-hybrid analysis, we focused on those colonies that were able to grow under the most stringent conditions tested (i.e. in the absence of histidine and presence of 10 mM 3AT or in the absence of adenine). Prey plasmid inserts from 40 colonies selected by this criterion were amplified by PCR and identified by sequencing. One insert corresponded to the full ORF of Dysbindin, which has previously been shown to interact with Pallidin in the yeast two-hybrid system (16). Another eight comprised the full ORF of BLOS1 (with variable lengths of 5'-untranslated region), and one contained a truncated version of BLOS1 comprising residues 79–125 (referred to herein as BLOS1ΔN). To verify the specificity of the yeast two-hybrid interaction between Pallidin and BLOS1, we generated Gal4 DNA-binding or activation domain constructs containing BLOS1 devoid of the 5'-untranslated region and subsequently assayed them for interaction with appropriate pallidin fusion constructs. As shown in Fig. 2, interaction between Pallidin and either full-length or truncated forms of BLOS1 could be demonstrated in both construct configurations (i.e. with pallidin fused to the DNA-binding (Fig. 2A) or activation (Fig. 2B) domains), and none of the fusion constructs caused spurious expression of the reporter gene when co-transformed with the corresponding empty vector. Although both BLOS1 and Pallidin bear regions predicted to form coiled-coil structures, the BLOS1 region seems to be dispensable for Pallidin binding, since it was absent from the BLOS1ΔN construct, which interacted with Pallidin.

Endogenously Expressed Snapin, BLOS1, BLOS2, and BLOS3 Are Subunits of BLOC-1—To test directly the idea that Snapin and the BLOS proteins are stable components of the complex, we generated affinity-purified rabbit polyclonal antibodies to each of them. By immunoblotting, each of the four antibodies recognized more than one protein in a crude cytosolic extract from mouse liver (Fig. 3A). To identify which of these proteins correspond to the bona fide antigens, we extended our analysis to extracts from pallid mice, which display no detectable Pallidin and drastically reduced steady-state levels of the Muted and Dysbindin proteins, probably due to partial destabilization and degradation of BLOC-1 (Refs. 16, 20, 21; see also Fig. 1A). Each antibody recognized a single protein band that was decreased or barely detectable in pallid mouse extracts.
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Fig. 2. Interaction between BLOS1 and Pallidin in the context of the yeast two-hybrid system. A and B, yeast cells were co-transformed with expression plasmids containing Gal4 DNA-binding and activation domains alone (vector) or fused in frame to full-length human Pallidin, full-length human BLOS1, or BLOS1 residues 79–143 of the yeast two-hybrid system. A, double transformants were spotted onto plates containing histidine (His) and then spottted onto plates containing the same medium (as a control) or selective medium lacking histidine and containing 1 mM 3AT. Notice the growth on selective medium of cells co-expressing Pallidin fused to the DNA-binding domain (A) or the activation domain (B) and the corresponding constructs containing BLOS1 or BLOS1ΔN.

Fig. 3. Immunochemical detection of endogenous Snapin, BLOS1, BLOS2, and BLOS3 from mouse liver and human HeLa cells. A, immunoblotting analysis of liver cytosolic extracts (~75 μg of total protein) from wild-type (C57BL/6J), pallid, and reduced pigmentation (Red. pigm.) mice, using antibodies to the indicated proteins. Notice the apparent absence of the Pallidin protein from the pallid liver extract, the reduced level of the same protein in the reduced pigmentation extract, and the reduced levels of the Snapin, BLOS1, and BLOS2 as well as the apparent lack of BLOS3 immunoreactivity in extracts from both pallid and reduced pigmentation mice. The antibodies to Snapin, BLOS1, BLOS2, and BLOS3 recognized additional proteins that were not decreased in pallid and reduced pigmentation extracts and were deemed to represent nonspecific cross-reactivity. The position of selected molecular mass standards are indicated on the right. B, immunoprecipitation-recapture of the Snapin, BLOS1, BLOS2, and BLOS3 proteins from human HeLa cells. Cells metabolically labeled with [35S]methionine and [35S]cysteine were lysed under non-denaturing conditions, and the cleared lysates were subjected to a first immunoprecipitation (1st IP) using irrelevant rabbit IgG or affinity-purified antibodies to Snapin, BLOS1, BLOS2, and BLOS3. Subsequently, the immunoprecipitates were denatured by heating in the presence of SDS and diethiothreitol, diluted in buffer, and subjected to a second immunoprecipitation (2nd IP) using the indicated antibodies. The final immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The positions of molecular mass standards are indicated on the left. The asterisk denotes a doublet of radiolabeled protein bands that were present in all samples and were deemed to represent nonspecific interactions with the antibody-containing beads.

(A) As an independent approach to identify endogenous Snapin, BLOS1, BLOS2, and BLOS3, the proteins were isolated from a metabolically labeled HeLa cell extract by a combination of non-denaturing immunoprecipitation followed by denaturation of the washed immunoprecipitates and a second (recapture) immunoprecipitation step. Here, the antigens were identified as radiolabeled proteins that were isolated when the specific antibodies were used in both immunoprecipitation steps, but not when the specific antibodies were replaced by irrelevant IgG at either step (Fig. 3B). Importantly, the electrophoretic mobilities of the endogenous antigens identified by the two approaches matched closely, except for a minor BLOS1 species that was detected by immunoprecipitation-recapture and not by immunoblotting (Fig. 3). The apparent molecular masses of Snapin (~15 kDa), BLOS1 (~13 kDa), and BLOS2 (~18 kDa) were in close agreement with the masses calculated from their predicted amino acid sequences (14.9, 14.3, and 16 kDa, respectively). On the other hand, the apparent size of the BLOS3 protein corresponded to a ~32-kDa polypeptide, larger than that predicted from the calculated molecular mass (21.3 and 20.4 kDa for the human and mouse proteins, respectively). Interestingly, these apparent molecular masses are consistent with the idea that the three main bands of 13, 15, and 18 kDa observed in the purified BLOC-1 preparation may represent BLOS1, Snapin, and BLOS2, respectively, and that a faint band of ~32 kDa may correspond to BLOS3 (Fig. 1C).

Next, we tested for stable association of candidate BLOC-1 subunits with known components of the complex by co-immunoprecipitation of endogenous proteins. To this end, BLOC-1 was immunoprecipitated from metabolically labeled HeLa cells using antibodies to Pallidin, Dysbindin, BLOS2, or BLOS3 (our antibodies to Muted, Snapin, and BLOS1 were also capable of immunoprecipitating the complex, albeit with lower efficiencies; data not shown). Subsequently, the complex was dissociated by denaturation in the presence of SDS and dithiothreitol, and the isolated subunits were “recaptured” in a second immunoprecipitation step using appropriate antibodies. Importantly, Pallidin, Muted, Dysbindin, and the four candidate subunits were all recovered in significant amounts from immunoprecipitated BLOC-1, regardless of which antibody (i.e. to Pallidin, Dysbindin, BLOS2, or BLOS3) was used to immunoprecipitate the whole complex in the first step (Fig. 4). As expected, none of the proteins were recovered from a first immunoprecipitation step using an irrelevant rabbit IgG (Fig. 4), and a subunit of another protein complex (the AP-4 ε subunit) was not detected in any of the first immunoprecipitates (data not shown).

Finally, we tested whether the endogenous Snapin and BLOS proteins co-fractionate with known BLOC-1 subunits during size exclusion chromatography, which would be expected if these proteins are stable components of the complex. To this end, a liver cytosolic extract from wild-type mice was fractionated on a Superose 6 column, and the resulting fractions were analyzed by immunoblotting. As shown in Fig. 5A, the bulk of Snapin and the BLOS proteins co-eluted with Pallidin and Dysbindin in fractions corresponding to a Stokes radius of ~94 Å, a value in close agreement with the previously estimated size of the complex (16, 20).

Taken together, all of the biochemical experiments described above strongly suggest that Snapin, BLOS1, BLOS2, and BLOS3 are stable components of BLOC-1.
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**Binary Interactions among BLOC-1 Subunits**—To examine direct contacts between Pallidin, Muted, Cappuccino, Dysbindin, and the four BLOC-1 subunits identified in this study, we tested 56 of 64 possible interactions (including the interaction of each subunit with itself) by means of the yeast two-hybrid system. The eight possible interactions that were not tested involved Dysbindin fused to the Gal4 DNA-binding domain, since this fusion construct caused activation of the reporter gene in the absence of any interaction partner (data not shown). As shown in Fig. 6A, 17 positive interactions were detected. Two of them, namely the interaction of Pallidin with itself and with Dysbindin, have been reported previously (16, 20, 21). The remaining 15 interactions correspond to four interaction pairs that yielded positive results in both construct configurations (i.e. Pallidin-Cappuccino, Pallidin-BLOS1, Cappuccino-BLOS2, and Snapin-BLOS2) and seven that were detected in only one configuration, three of them because they involved Dysbindin and could only be tested using this protein fused to the activation domain. In additional control experiments (not shown), none of the BLOC-1 subunits interacted with a fusion construct containing the coiled-coil region of HPS1, which is a component of BLOC-3 (25–27). The interaction network inferred from this analysis (Fig. 6B) seems to be well connected, with six of the eight subunits involved in at least three intersubunit interactions.

**The BLOS3-encoding Gene Is Mutated in Reduced Pigmentation Mice**—The genes encoding previously known BLOC-1 subunits, Pallidin, Muted, Cappuccino, and Dysbindin, are mutated in mouse models of HPS (16, 22, 28, 29). We therefore considered the possibility that one or more of the genes encoding the newly identified BLOC-1 subunits could be defective in additional mouse models of the disease. In particular, previous studies (20, 21) have established that the steady-state levels of Pallidin and Muted are drastically reduced in cell extracts from the mutant mouse strain, reduced pigmentation (rp), for which the defective gene has been mapped to chromosome 7A2 (2.0 centimorgans) by linkage analysis but not yet identified (42). Although one study reported normal levels of the Dysbindin protein in rp kidney extracts (16), we observed significantly reduced Dysbindin protein levels in cytosolic extracts from rp liver (Fig. 1A). Furthermore, we found decreased steady-state levels of the Snapin, BLOS1, and BLOS2 proteins and no detectable BLOS3 protein in liver extracts prepared from this mouse strain (Fig. 3A). Together, these results suggest that the product of the gene mutated in rp mice is required to maintain normal BLOC-1 protein levels.

Data base searching revealed that the mouse BLOS3 is identical to hypothetical protein E230011O18 (RefSeq Accession number NP_808360), which has been mapped to mouse chromosome 7A2 (16.5 megabase pairs) within the chromosomal region predicted to contain the gene defective in rp mice (Mouse Genome Informatics database, available on the World Wide Web at www.informatics.jax.org) (43). To test whether rp mice carry deleterious mutations in the BLOS3-encoding gene, total RNA was extracted from skin fibroblasts and liver of wild-type and mutant animals and used for mutation screening and real time PCR analysis. For screening of mutations that would alter the primary structure of BLOS3, the full-length ORF was amplified by RT-PCR and sequenced. A single nucleotide substi-
A single nucleotide substitution resulting in an early termination codon in BLOS3 from reduced pigmentation mice. The results are representative of the analysis of three animals of each strain. C, coat color phenotypes of mice belonging to the control C57BL/6J strain (wild type) and the congenic mutant strains, reduced pigmentation and pallid.

**Fig. 7. Reduced pigmentation mice carry a nonsense mutation in the gene encoding BLOS3.** A and B, relevant portions of electrophoregrams resulting from sequencing the ORF of BLOS3 cDNAs obtained from fibroblasts of C57BL/6J (A) and reduced pigmentation (B) mice. Notice the presence of a single nucleotide substitution resulting in an early termination codon in BLOS3 from reduced pigmentation mice. The results are representative of the analysis of three animals of each strain. C, coat color phenotypes of mice belonging to the control C57BL/6J strain (wild type) and the congenic mutant strains, reduced pigmentation and pallid.

**DISCUSSION**

BLOC-1 was originally described as a multisubunit protein complex containing the Pallidin and Muted polypeptides as...
Identification of Previously Unknown Subunits of BLOC-1

well as an undetermined number of additional components (20, 21). Subsequently, Cappuccino and Dysbindin were also identified as BLOC-1 subunits (16, 22). The four proteins are small (20–40 kDa) and contain regions predicted to adopt a coiled-coil structure. Pallidin was reported to interact with Syntaxin 13, a SNARE protein localized to early endosomes and implicated in membrane fusion (28). Dysbindin was originally described as a binding partner of α- and β-dystrobrevins (two proteins related to the dystrophin protein associated with Duchenne/Becker muscular dystrophy) and recently reported to interact with a novel, muscle-specific protein named Myospryn (30, 44). The physiological significance of these interactions remains to be determined.

In this work, we have identified four previously unknown subunits of BLOC-1: Snapin, BLOS1, BLOS2, and BLOS3. The four proteins were detected by mass spectrometry-based analysis of partially purified BLOC-1 from bovine liver, and BLOS1 was also isolated through a yeast two-hybrid screen for Pallidin-binding proteins. Several lines of evidence demonstrate that Snapin, BLOS1, BLOS2, and BLOS3 are all subunits of BLOC-1. First, the endogenous steady-state levels of the four proteins were significantly reduced in cells from pallid mice, which lack detectable Pallidin and display reduced levels of other BLOC-1 components. Second, the four proteins co-immunoprecipitated with Pallidin, Muted, and Dysbindin upon immunoprecipitation-recapture of metabolically labeled HeLa cells. Third, the four proteins co-fractionated with Pallidin and Dysbindin upon size exclusion chromatography of mouse liver cytosol. Finally, the four proteins engage in a network of binary interactions that also involves all of the previously known BLOC-1 subunits, as inferred from yeast two-hybrid experiments. Interestingly, a recent genome-wide analysis of the interaction map of Drosophila melanogaster (45) identified interactions between the Drosophila counterparts of Dysbindin (CG6856) and Pallidin (CG14133), Pallidin and BLOS1 (CG30077), and Snapin (CG32951) and BLOS2 (CG14145); these reported interactions are fully consistent with our results shown in Fig. 6. Taken together, our data strongly suggest that Snapin, BLOS1, BLOS2, and BLOS3 are part of BLOC-1. However, we cannot rule out the possibility that some of these proteins may have additional physiological functions independent of the complex.

Have all BLOC-1 subunits been identified? Based on the hydrodynamic properties of the complex, the native molecular mass was previously calculated to be 200 ± 30 kDa for bovine BLOC-1 (20) and ~230 kDa for mouse BLOC-1 (16). If the complex contains one copy of each of the eight known subunits, then the calculated molecular mass would be ~170 kDa. It is, therefore, possible that BLOC-1 contains more than one copy of some of the known subunits or additional subunits that remain to be identified.

Our finding that Snapin is a stable component of BLOC-1 provides new insights into the possible molecular function of BLOC-1. Snapin was first characterized as a SNAP-25-binding protein and proposed to regulate association of a core complex containing SNAP-25 and other SNARE proteins with synaptotagmin, a calcium-dependent activator of synaptic vesicle exocytosis (31) (see also Ref. 46). Although Snapin was originally described as a brain-specific, integral membrane protein (31), it was subsequently found (32) to be a ubiquitously expressed, soluble protein that is capable of associating with membranes and interacting with SNAP-23, a ubiquitous paralog of SNAP-25. The fact that we have isolated Snapin from bovine liver cytosol and immunochemically detected it in extracts from mouse liver and human HeLa cells is in agreement with the second study. In any case, the reported abilities of Snapin to interact with SNAP-25/23 (31, 32) and of Pallidin to interact with Syntaxin 13 (21, 28) argue for a role of BLOC-1 in the regulation of SNARE-mediated membrane fusion. Specifically, association of SNAP-25 with Syntaxin 13 on early endosomes has been demonstrated and proposed to represent an intermediate step in the formation of a fusogenic complex containing SNAP-25, Syntaxin 13, and VAMP-2 (47). Along these lines, it is tempting to speculate that BLOC-1 may regulate SNARE complex formation at some step in the endocytic pathway, as has been proposed for hepatocyte growth factor-regulated tyrosine kinase substrate and the homotypic fusion and vacuole protein sorting complex (47, 48). Such a hypothetical role for BLOC-1 would be consistent with its requirement for normal biogenesis of melanosomes and platelet dense granules, since the formation of these and other lysosome-related organelles is thought to involve one or more endosomal compartments. Nevertheless, it is clear that future work is required to ascertain the molecular function of BLOC-1.

Each of the previously known BLOC-1 subunits is mutated in a distinct mouse model of HPS, and mutation of human Dysbindin causes HPS type 7 (16, 22, 28, 29). Here we report that the gene encoding BLOS3 is mutated in the rp mouse. The detected mutation is a single nucleotide substitution within the coding region and results in a premature stop codon. Cells from rp mice contain no detectable BLOS3 and display significantly reduced levels of all other BLOC-1 subunits. These results are consistent with the idea that the BLOS3-encoding gene is the target of the rp mutation and that the defect in BLOS3 causes secondary destabilization and partial loss of BLOC-1 subunits. However, at this point, we cannot completely rule out the possible existence of a second mutation in a neighboring gene contributing to the rp phenotype. Future experiments, such as phenotype rescue of rp mice by transgenic technology or targeted disruption of the BLOS3-encoding gene in wild-type mice, will be necessary to address this issue.

The coat color phenotype of homozygous rp mice is less severe than that of homozygous pallid mice. This phenotypic difference correlates with the extent of assembly of BLOC-1 subunits, as inferred from size exclusion chromatographic analysis of liver cytosolic extracts prepared from these mutant mice. Unlike the chromatographic behavior of Dysbindin, Snapin, and BLOS1 from pallid cytosol, which appeared to associate into at least two BLOC-1 subcomplexes, the three proteins and Pallidin from rp cytosol significantly co-eluted in fractions corresponding to a size slightly smaller than that of native BLOC-1. A likely interpretation for these results is that the rp mutation is functionally hypomorphic, allowing the residual complex to provide some biological activity. One may speculate either that the structural role of BLOS3 in BLOC-1 assembly is relatively less important than that of Pallidin or that a truncated BLOS3 polypeptide is expressed in rp mice that can associate with the other BLOC-1 subunits. The first possibility is consistent with the idea that BLOS3 may not be a part of the complex core, as suggested by the fact that only one interaction involving BLOS3 was detected in our yeast two-hybrid analysis (Fig. 6). Furthermore, an antibody raised to the amino-terminal region of BLOS3 failed to detect any truncated form of BLOS3 in rp cells, under conditions in which it readily detected the BLOS3 protein from wild-type cells (data not shown).

In summary, we have identified Snapin and three formerly uncharacterized proteins (BLOS1, BLOS2, and BLOS3) as subunits of BLOC-1 and found that the HPS mouse model strain, reduced pigmentation, carries a nonsense mutation in BLOS3. The genes encoding these proteins may underlie the pathogenesis of novel forms of HPS in patients having no detectable mutation in any of the genes that cause HPS types 1–7.
Acknowledgments—We are indebted to Cristina A. Ghiani, Vincent Lelièvre, and Jean S. de Vellis for helping with the real time PCR analysis. We thank Ramin Nazarian for excellent technical assistance and Cristina A. Ghiani, David E. Krantz, Gregory S. Payne, and the members of our laboratory for critical reading of the manuscript.

REFERENCES

1. Huizing, M., Boissy, R. E., and Gahl, W. A. (2002) Pigment Cell Res. 15, 405–419
2. Starievic, M., Nazarian, R., and Dell'Angelica, E. C. (2002) Semin. Cell Dev. Biol. 13, 271–278
3. Spritz, R. A., Chiang, P.-W., Oiso, N., and Alkhateeb, A. (2003) Curr. Opin. Genet. Dev. 13, 284–289
4. Dell'Angelica, E. C., Mullins, C., Caplan, S., and Sofroniew, M. V. (2000) FASEB J. 14, 1265–1278
5. Marks, M. S., and Seabra, M. C. (2001) Nat. Rev. Mol. Cell Biol. 2, 738–748
6. King, S. M., and Reed, G. L. (2002) Mol. Cell. Biol. 22, 389–392
7. Swank, R. T., Novak, E. K., McGarry, M. P., Rusiniak, M. E., and Fung, L. (1998) Pigment. Cell Res. 11, 60–80
8. Nakatani, Y., Nakamura, N., Sano, J., Inayama, Y., Kawano, N., Yamanaka, S., Miyagi, Y., Nagashima, Y., Ohbayashi, C., Mizushima, M., Manabe, T., Kuroda, M., Yokoi, T., and Matsubara, O. (2000) Virchows Arch. 437, 304–313
9. Lyerla, T. A., Rusiniak, M. E., Borchers, M., Jahreis, G., Tan, J., Ohtake, P., Novak, E. K., and Swank, R. T. (2003) Am. J. Physiol. 285, L643–L653
10. Clark, R. H., Stinchcombe, J. C., Day, A., Blott, E., Bossi, G., Hamblin, T., Davies, E. G., and Griffiths, G. M. (2003) Nat. Immunol. 4, 1111–1120
11. Oh, J., Bailin, T., Fukai, K., Feng, G. H., Ho, L. M., Zhao, J. L., Frenk, E., Yamagata, N., and Spritz, R. A. (1996) Nat. Genet. 14, 300–306
12. Dell'Angelica, E. C., Shotelersuk, V., Aguilar, R. C., Gahl, W. A., and Bonifacino, J. S. (1999) Mol. Cell 3, 11–21
13. Anukeste, Y., Huizing, M., White, J., Shevchenko, Y. O., Fitzpatrick, D. L., Touchman, J. W., Compton, J. G., Bale, S. J., Swank, R. T., Gahl, W. A., and Torro, J. R. (2001) Nat. Genet. 28, 376–380
14. Suzuki, T., Li, W., Zhang, Q., Karim, A., Novak, E. K., Sviderskaya, E. V., Hill, S. P., Bennett, D. C., Levin, A. V., Nieuwenhuis, H. K., Fong, C.-T., Casper, R. F., Touchman, J. W., Compton, J. G., Bale, S. J., Swank, R. T., Gahl, W. A., and Torro, J. R. (2001) Nat. Genet. 28, 376–380
15. Zhang, Q., Zhao, B., Li, W., Oiso, N., Novak, E. K., Rusiniak, M. E., Gautam, R., Chintala, S., O'Brien, E. P., Zhang, Y., Roe, B. A., Elliott, R. W., Eicher, E. M., Liang, P., Kratz, C., Legius, E., Spritz, R. A., O'Sullivan, T. N., Copeland, N. G., Jenkins, N. A., and Swank, R. T. (2002) Nat. Genet. 33, 145–153
16. Li, W., Zhang, Q., Oiso, N., Novak, E. K., Gautam, R., O'Brien, E. P., Tinsley, C. L., Blake, D. J., Spritz, R. A., Copeland, N. G., Jenkins, N. A., Amato, D., Roe, B. A., Starievic, M., Dell'Angelica, E. C., Elliott, R. W., Mishra, V., Sviderskaya, E. V., Hill, S. P., Bennett, D. C., Levin, A. V., Nieuwenhuis, H. K., Fong, C.-T., Castellan, C., Mitermski, B., Swank, R. T., and Spritz, R. A. (2002) Nat. Genet. 32, 321–324
17. Robinson, M. S., and Bonifacino, J. S. (2001) Curr. Opin. Cell Biol. 13, 444–453
18. Banta, L. M., Vida, T. A., Herman, P. K., and Emr, S. D. (1990) Mol. Cell. Biol. 10, 4638–4649
19. Seals, D. F., Fitzen, G., Margolis, N., Wickner, W. T., and Price, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9402–9407
20. Falco-Pérez, J. M., Starievic, M., Gautam, R., and Dell'Angelica, E. C. (2002) J. Biol. Chem. 277, 28191–28199