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# A divalent interaction between HPS1 and HPS4 is required for the formation of the biogenesis of lysosome-related organelle complex-3 (BLOC-3)

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ABSTRACT

Hermansky–Pudlak syndrome (HPS) is a group of rare autosomal recessive disorders characterized by oculocutaneous albinism, a bleeding tendency, and sporadic pulmonary fibrosis, granulomatous colitis or infections. Nine HPS-causing genes have been identified in humans. HPS-1 is the most severe subtype with a prevalence of ~1/1800 in northwest Puerto Rico due to a founder mutation in the *HPS1* gene. Mutations in HPS genes affect the biogenesis of lysosome-related organelles such as melanosomes in melanocytes and platelet dense granules. Two of these genes (*HPS1* and *HPS4*) encode the HPS1 and HPS4 proteins, which assemble to form a complex known as Biogenesis of Lysosome-related Organelle Complex 3 (BLOC-3). We report the identification of the interacting regions in HPS1 and HPS4 required for the formation of this complex. Two regions in HPS1, spanning amino acids 1–249 and 506–700 are required for binding to HPS4; the middle portion of HPS1 (residues 250–505) is not required for this interaction. Further interaction studies showed that the N-termini of HPS1 and HPS4 interact with each other and that a discrete region of HPS4 (residues 340–528) interacts with both the N- and C-termini of the HPS1 protein. Several missense mutations found in HPS-1 patients did not affect interaction with HPS4, but some mutations involving regions interacting with HPS4 caused instability of HPS1. These observations extend our understanding of BLOC-3 assembly and represent an important first step in the identification of domains responsible for the biogenesis of lysosome-related organelles.


## 1. Introduction

Hermansky–Pudlak syndrome (HPS) [MIM# 203300] is a group of autosomal recessive conditions characterized by hypopigmentation and a bleeding tendency caused by defective biogenesis of lysosome-related organelles such as melanosomes and platelet dense granules [1–4]. Some HPS subtypes develop other complications such as pulmonary fibrosis and/or granulomatous colitis [3]. In humans, nine different genes have been found to cause HPS but in mouse, mutations in at least 16 genes cause HPS-like phenotypes [5–12]. The majority of these genes encode proteins of unknown structure and function. Others encode subunits of well-characterized complexes involved in vesicular trafficking such as AP-3 and HOPS [6]. HPS gene products have been identified as subunits of at least three multi-protein complexes named BLOC (**B**iogenesis of **L**ysosome-related **O**rganelles **C**omplex) -1 to -3. The precise functions of BLOC complexes have not been well established, although physical interactions between components of these complexes have been suggested and defined [13].

In particular, BLOC-3 consists of two cytosolic proteins, HPS1 and HPS4 [14–17].

Patients with mutations in either subunit of the heterodimer generally develop fatal lung fibrosis in their fourth to fifth decade of life or earlier in some cases [3]. A frameshift mutation consisting of duplication of a 16 base pairs sequence in exon 15 of the *HPS1* gene is the most common mutation in the northwest region of the island of Puerto Rico [5]. The *HPS1* gene encodes a 700 amino acids-long protein with a molecular weight of 79.3 kDa [5], whereas *HPS4* codes for a 76.9-kDa polypeptide of 708 amino acids. Both proteins exhibit a cytosolic distribution with approximately 10% associated to membranes, but association to a specific cellular organelle has not been established [14–16]. Co-immunoprecipitation of epitope-tagged and endogenous proteins demonstrated a tight interaction between these two proteins. However, this interaction was not evident on yeast two hybrid analysis, which suggested that additional components of BLOC-3 may exist [14–16]. Rab9, a small GTPase that localizes in late endosomes, was recently shown to interact with BLOC-3 [17]. Bioinformatic analyses have suggested that HPS proteins exhibit

*Abbreviations:* BLOC, biogenesis of lysosome-related organelles; DUF, domain of unknown function; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; HA, hemagglutinin; HPS, Hermansky–Pudlak syndrome; IgG, immunoglobulin G; LAMP-2, lysosome-associated membrane protein 2; NaCl, sodium chloride; PAT, perilipin adipophilin, TIP47; RT-PCR, reverse transcriptase-polymerase chain reaction.

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some homology to yeast proteins, including some that participate in intracellular vesicular trafficking [18,19]. In particular, HPS4 contains a conserved N-terminal domain of approximately 200 amino acids, which has been termed CHiPS (for *CCZ1–HPS4*) [18]. In addition, a central region of approximately 200 residues of HPS1 has homology to the C-terminal domain of members of the PAT family (for Perilipin, Adipophilin, and TIP47 (tail-interacting protein of 47 kDa) [18]. In order to elucidate and understand the molecular function of the BLOC-3 complex, it is important to assess the organization and interacting regions critical for complex formation.

In this study, we identified the regions in HPS1 and HPS4 required for the formation of BLOC-3. Point mutations in the interacting regions destabilize the proteins, suggesting that they are important for complex stability. These observations extend our understanding of BLOC-3 assembly and represent an important first step in identifying novel protein domains responsible for the biogenesis of lysosome-related organelles.

## 2. Materials and methods

### 2.1. Antibodies

The following mouse monoclonal antibodies were used: anti-Myc (9E10), anti-GFP (B-2) and anti-V5 (Santa Cruz Biotechnology), hemagglutinin (HA) tag (HA.11; Covance, Princeton, NJ), anti-Akt, anti-Phospho Akt (Cell Signaling Technology, Beverly, MA), anti-HPS1 (hHPS5, a gift of Dr. Richard Spritz, University of Colorado) and the rabbit polyclonal antibody anti-LAMP2 (Abcam), anti-HPS4 (a gift of Dr. Juan Bonifacino). Donkey Alexa-488 anti-mouse IgG and Alexa-555 anti rabbit IgG were purchased from Molecular probes (Eugene, OR). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs were purchased from Amersham Biosciences, Piscataway, NJ.

### 2.2. Cell culture and transfections

Normal (GM00037H) and Puerto Rican HPS1 16 bp duplication mutant fibroblasts (GM14609) were obtained from the Coriell Institute for Medical Research and maintained in MEM Eagle-Earle supplemented with 15% fetal bovine serum (FBS). HeLa and M1 cells (a gift of Dr. Juan Bonifacino, NICHD) were maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen). For the stably transfected M1 clone 26, expressing $Myc_3$–HPS4, the medium was supplemented with 600 μg/ml G-418. All cell cultures were supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and grown in a humidified incubator with 5% $CO_2$ at 37 °C. Transfections were carried out using the Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions.

### 2.3. cDNA tissue panel PCR

For tissue-specific expression studies of alternatively spliced RNA isoforms, human multiple tissue cDNA panels (human MTC panels I and II) were purchased from Clontech/BD Biosciences. We designed a primer set for PCR amplification of the regions where the splice variant(s) differed and generated products of different lengths. The forward primer 5′-GGCAACTTCCTGTATGTCCTTCACCTG-3′ was located before exon 5 and the reverse primer 5′-CTGCTATCTGAAGGGC ATCC-3′ hybridizes after exon 9.

### 2.4. DNA constructs and mutations

The pCI–$HA_3$–HPS1 and pCI–$Myc_3$–HPS4 vectors were generated as described [14]. The full length coding sequence of *HPS1* was obtained by PCR amplification of cDNA and cloned in frame into the *Xho1* and *EcoR1* sites of the *pEGFP1-C1* vector (BD Biosciences Clontech, Palo Alto, CA). Mutagenesis of expression constructs was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) as recommended by the manufacturer. To generate the pCI-$Myc_3$–$HPS4^{340–708}$ and pCI-$Myc_3$–$HPS4^{528–708}$ constructs, the desired portions of the HPS4 coding region were amplified by PCR using the pCI-$Myc_3$–HPS4 plasmid as template. PCR products were sub-cloned into the *Eco*RI and *Xba*I sites of the pCI-$Myc_3$ vector. All the constructs were sequenced on both strands to confirm their identity and the presence of the desired mutations, if applicable. Sequencing was performed using ABI BigDye Terminator chemistry (Applied Biosystems, Foster City, CA) with detection on an ABI 310 or a 3130xl Genetic Analyzer (Applied Biosystems). Electrophoregrams were analyzed using Sequencher v4.9 software (Gene Codes Corporation, Ann Arbor, MI).

### 2.5. Co-immunoprecipitations and Western blotting

Transfected cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and incubated in 400 μl of lysis buffer [50 mM Tris–HCl pH 7.4, 300 mM NaCl, 0.5% w/v Triton X-100, 5 mM EDTA, 0.1% BSA, 1× protease inhibitor cocktail (Roche, Indianapolis, IN)]. After 30 min of incubation at 4 °C, lysates were centrifuged at 16,000 ×*g* for 15 min. The supernatants were then pre-cleared by incubation for 60 min at 4 °C with G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The pre-cleared lysates were subsequently incubated overnight at 4 °C with G-Sepharose beads and mouse monoclonal antibody against the specific tag. The beads were washed three times with 1 ml ice-cold lysis buffer and once with ice-cold PBS. Bound proteins were eluted by boiling in 30 μl Laemmli buffer at 95 °C for 5 min. Samples were analyzed by SDS-PAGE and immunoblotting. SDS–PAGE analysis and electroblotting onto nitrocellulose membranes was performed using the NuPAGE® Bis-Tris Gel system (Invitrogen), according to the manufacturer's instructions. Nitrocellulose membranes were incubated with primary and horseradish peroxidase-labeled secondary antibodies and reactive proteins were detected using ECL Western Blotting Substrate from Pierce (Rockford, IL).

### 2.6. Cell fractionation

To prepare cytosolic and membrane fractions, transfected cells were washed twice in phosphate-buffered saline (PBS), detached by scraping, suspended in buffer A (25 mM HEPES pH 7.4, 0.25 M sucrose, 1 mM EGTA, 0.5 EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail). Cells were mechanically disrupted by successive passages through a 25-gauge needle. Extracts were centrifuged at 800 ×g for 10 min, and the resulting post-nuclear supernatants were then centrifuged at 120,000 ×*g* for 45 min at 4 °C to yield cytosolic and membrane fractions. Membranes were resuspended in equal volume of buffer A containing 0.1% Triton-X100. Both fractions were analyzed by immunoblotting.

### 2.7. Pulse-chase assay

The pEGFP-C1-HPS1 and HPS1 mutant constructs were transfected in M1 cells using 1 μg of each plasmid. One day after transfection, cells were washed with PBS and translation was inhibited using 100 μg/mL cycloheximide and 40 μg/mL chloramphenicol in 1 mL of media. Samples were then collected at 0, 1, 3, and 6 h after translation inhibition. Collected cells were lysed using 200 μl of lysis buffer. Equal volumes of each sample were analyzed by SDS-PAGE and immunoblotting.

### 2.8. Immunofluorescence Analysis

Transfected M1 cells were washed twice with PBS containing $Ca^{+2}/Mg^{+2}$, fixed in 4% PFA in PBS, and permeabilized for 10 min with 0.2% (wt/vol) Triton X-100 in PBS. After permeabilization, cells were blocked for 30 min with 0.2% (wt/vol) porcine skin gelatin in PBS and incubated in a humid chamber for 1 h at 37 °C with