The Hermansky-Pudlak Syndrome 1 (HPS1) and HPS4 Proteins are Components of two Complexes, BLOC-3 and BLOC-4, Involved in the Biogenesis of Lysosome-Related Organelles*

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Running title: Hermansky-Pudlak syndrome 1 and 4 protein complexes

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SUMMARY

Hermansky-Pudlak syndrome (HPS)\(^1\) is a genetic disease of lysosome, melanosome, and granule biogenesis. Mutations of six different loci have been associated with HPS in humans, of which mutations of the \(HPS1\) and \(HPS4\) genes are the most frequent. Here, we show that the HPS1 and HPS4 proteins are components of two novel protein complexes involved in biogenesis of melanosome and lysosome-related organelles, BLOC-3 (biogenesis of lysosome-related organelles complex 3) and BLOC-4. The phenotypes of \(Hps1\)-mutant (pale-ear; \(ep\)) and \(Hps4\)-mutant (light-ear; \(le\)) mice and humans are very similar, and cells from these mice exhibit similar abnormalities of melanosome morphology. HPS1 protein is absent from \(ep\)-mutant cells, and HPS4 from \(le\)-mutant cells, but \(le\)-mutant cells also lack HPS1 protein. HPS4 protein appears necessary for stabilization of HPS1, and the HPS1 and HPS4 proteins co-immunoprecipitate, indicating they are in a complex. HPS1 and HPS4 do not interact directly in a yeast 2-hybrid system, although HPS4 interacts with itself. In a partially purified vesicular/organellar fraction, HPS1 and HPS4 are both components of a \(~500\) kDa M.W. complex, termed BLOC-3. Within BLOC-3, HPS1 and HPS4 are components of a discrete \(~200\) kDa module, termed BLOC-4. In the cytosol, HPS1 (but not HPS4) is part of yet another complex, termed BLOC-5. We propose that the BLOC-3 and BLOC-4 HPS1-HPS4 complexes play a central role in trafficking cargo proteins to newly-formed cytoplasmic organelles.
Hermansky-Pudlak syndrome (HPS; MIM 203300) is a group of autosomal recessive genetic diseases characterized by pulmonary fibrosis, oculocutaneous albinism, bleeding tendency, and progressive pulmonary fibrosis, the result of defective biosynthesis of lysosomes, melanosomes, and platelet dense granules (1-3). In humans, HPS has been associated with defects in six different genes (4-8); in mice, at least 16 different loci with HPS-like mutant phenotypes are known (9, 10). Several of these mouse HPS model strains bear mutations in orthologues of the human HPS genes (7, 8, 11-14). Five of the various human and mouse HPS-related loci encode proteins that regulate vesicular protein trafficking, including \( \text{AP3B1} \) (mouse pearl, \( \text{pe} \); human HPS2, AP-3 adaptin \( \beta \) subunit; 13), \( \text{AP3D1} \) delta (mouse mocha, \( \text{mh} \); AP-3 adaptin \( \delta \) subunit; 15), \( \text{RAB27A} \) (mouse ashen, \( \text{ash} \); human Griscelli syndrome; Rab27A; 16), \( \text{RABGGTA} \) (mouse gunmetal, \( \text{gm} \); Rab geranylgeranyl transferase \( \alpha \) subunit; 17), and \( \text{VPS33A} \) (mouse buff, \( \text{bf} \); class C vacuole/pre-vaculole t-SNARE complex subunit; 18). However, the great majority of the human and mouse HPS-related genes encode proteins whose functions are not yet known.

Most human HPS patients have mutations of either \( \text{HPS1} \) or \( \text{HPS4} \). Human HPS1 is a 700-amino acid, 79.3 kDa, non-membrane protein, and HPS4 a 708-amino acid, 76.9 kDa, non-membrane protein, both unrelated to other known proteins and containing no sequence motifs that might provide clues to their functions in organellar biogenesis. Nevertheless, several lines of evidence suggest that the HPS1 and HPS4 proteins may be functionally related. First, \( \text{Hps1} \)-mutant (pale-ear; \( \text{ep} \)) and \( \text{Hps4} \)-mutant (light-ear; \( \text{le} \)) mice exhibit very similar phenotypes, grossly and at the biochemical and cellular ultrastructural levels (19-21). Second, \( \text{eplep} \), \( \text{lelle} \) double-homozygous mutant mice have a phenotype that is the same as that of the two homozygous single mutants (22). Third, the HPS1 protein is absent in cells of both \( \text{Hps1} \)-
mutant (ep) and Hps4-mutant (le) mice (7). Finally, the HPS1 and HPS4 proteins partially co-localize in cytoplasmic vesicles of transfected melanoma cells (7). Together, these data strongly suggest a functional relationship or interaction between the HPS1 and HPS4 proteins.

Here, we show that the human HPS1 and human HPS4 proteins are both components of a ~500 kDa M.W. complex associated with vesicles and organelles, which we named BLOC-3 (biogenesis of lysosome-related organelles complex 2). Within BLOC-3, HPS1 and HPS4 are contained in a discrete ~200 kDa M.W. module, which we term BLOC-4. It is likely that BLOC-4, and certainly BLOC-3, contain additional protein components not yet identified. Our findings indicate that, as components of BLOC-3 and BLOC-4, the HPS1 and HPS4 proteins play a coordinated role in trafficking of proteins to newly-synthesized organelles.

**EXPERIMENTAL PROCEDURES**

*Dna constructs*—Full-length human *HPS1* (4) and *HPS4* (7) cDNAs were cloned in the Gateway Cloning Technology (Invitrogen) entry vector pENTR3C, and the *HPS1* and *HPS4* cDNA cassettes were then transferred to pDEST26, a vector for N-terminal His-fusion protein expression, and subsequently to the mammalian expression vector pIREShyg2 (Invitrogen), creating pIREShyg2-*HPS1* and pIREShyg2-*HPS4*, respectively. To express GST-HPS4 fusion protein in E. coli, we recombined pENTR3C-*HPS4* with pDEST15, a vector for N-terminal GST fusion expression from a T7 promoter, creating pDEST15-*HPS4*.

*Recombinant Protein Expression, Purification, and Antibody Production*—pDEST15-*HPS4* was introduced into *E. coli* BL21-CodonPlus-RP (Stratagene), and GST-HPS4 fusion protein was purified from inclusion bodies (23), and used as immunogen to prepare anti-hHPS4
polyclonal antisera in rabbits and HPS4 monoclonal antibodies in mice (Covance). Monoclonal antibodies to HPS1 were described previously (24). Monoclonal antibody to His-Tag was from Clontech.

Derivation and Transfection of ep- and le-Mutant Mouse Melanocytes—The wild-type mouse melanocyte line, melan-a (25) and melan-ep and melan-le melanocytes (7) have been described previously. Cells were cultured in RPMI1640 (InVitrogen), supplemented with 10% heat-inactivated FBS (HyClone), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 200 nM TPA at 37 °C in a 10% CO2 atmosphere. Melan-ep or melan-le melanocytes were transfected with the pIREShyg2-HPS1 and pIREShyg2-HPS4 constructs using FuGENE 6 Transfection Reagent (Roche), and stable transformants were selected in culture media containing 300 µg/ml hygromycin B. Human MNT1 melanoma cells (26) were cultured in DMEM (InVitrogen) supplemented with 20% heat-inactivated FBS, 10% AIM-V (PAN), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 100 nM Na-pyruvate at 37 °C in a 5% CO2 atmosphere. Cells were visualized by standard brightfield and Hoffman illumination microscopy.

Immunoblotting—Proteins were separated in 7.5% SDS-polyacrylamide and transferred to Immobilon–P membranes (Millipore). Membranes were blocked in 5% fat-free milk in PBS for 1 h at room temperature, and then were incubated in PBS containing 3% BSA, 0.2% Tween-20, and the appropriate primary antibody for 1 h at room temperature. Membranes were then washed extensively, incubated with appropriate HRP-conjugated secondary antibody (Pierce) for 1 h at room temperature, and washed again. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Blots were calibrated with pre-stained molecular
weight standards (Invitrogen). For analysis of HPS4-HPS1 complexes, immunoblots of gel filtration fractions, previously probed with anti-HPS1 antibody (24), were stripped in 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7 for 30 min at 50 °C, and were re-probed as described above.

**RT-PCR**—RNA from melan-a and melan-le melanocytes was isolated using the TRIzol reagent (Invitrogen). First strand cDNA was synthesized using SuperScript II RNase H- reverse transcriptase and oligo(dT)25 as primer (Invitrogen). PCR was performed using equivalent amounts of melan-a and melan-le RNAs, Platinum Taq DNA polymerase (Invitrogen), and mouse Hps1 forward (5’-GACTTCCGCTGCTACTTCCTACTTTCT-3’) and reverse (5’-GCGTGAGCAGCTCATAGCAC-3’) primers derived from exons 20 and 22, respectively.

**Coimmunoprecipitation Assays**—MNT1 cells were lysed in 50 mM Na-phosphate buffer (pH 8.0), 300 mM NaCl, 1% Tween-20, and protease inhibitors (Roche). 600 µl fresh lysate were incubated with 40 µl rabbit anti-HPS4 antisera, 40 µl HPS1 monoclonal antibody, 40 µl rabbit preimmune immunoglobulins (Santa Cruz), or 40 µl mouse preimmune immunoglobulins (Santa Cruz) for 1 hr at 4 °C with gentle agitation. Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia), pre-washed in lysis buffer, was added and the samples were incubated for 1 h at 4 °C. Immunoprecipitates were washed several times in lysis buffer and then boiled for 5 min in Laemmli sample buffer.

To test the effect of Tris-HCl on the BLOC-3 complex, we purified the vesicular-organellar fraction from MNT1 cells using as described (24) using 50 mM phosphate buffer (pH 8.0) as above. The fraction was then resuspended in either phosphate buffer or Tris buffer (0.25 M
sucrose, 100 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1% Tween-20), immunoprecipitated using HPS1 antibody or HPS4 antibody, and analyzed by western blot analysis using HPS4 antibody or HPS1 antibody, respectively.

For purification of His-tagged proteins, cells were lysed in PBS containing 1% NP40 and protease inhibitors, and lysates were incubated with TALON Metal Affinity Resins (Clontech) at room temperature for 30 min, washed several times in lysis buffer, and boiled for 5 min in Laemmli sample buffer.

**Yeast Two-Hybrid Assays**—Full length mouse **Hps1** and **Hps4** cDNAs were inserted in-frame in the DNA binding domain (pGBKTK7) and activation domain (pGADT7) Gal4 transcription factor vectors of the Matchmaker Gal4 Two-Hybrid System 3 (Clontech), and verified by sequencing. Fusion constructs were co-transformed into *Saccharomyces cerevisiae* strain AH109. At least two colonies of each double transformant growing on low stringency medium (-Trp/-Leu) were suspended in low stringency broth and grown at 30 ºC for 16 hr with shaking. Cell cultures were adjusted to 0.4 OD at 600 nm, and 3 µl from each co-transformation were spotted onto low and high stringency (-Trp/-Leu/-His/-Ade) plates containing X-alpha Gal. Plates were incubated at 30 ºC for 5 days and monitored for growth and blue color by visual inspection.

**RESULTS**

**Complementation Analysis of HPS1-Null and HPS4-Null Mouse Melanocytes**—We generated cultured melanocyte cell lines from homozygous **Hps1** null-mutant (*ep*) and **Hps4** null-mutant (*le*) mice, designated melan-ep and melan-le, respectively (7). As shown in Fig. 1, compared to wild-type melan-a melanocytes, both melan-ep and melan-le melanocytes appear
hypopigmented, contain relatively few melanized melanosomes, and show a wide range of melanosome sizes, including macro-melanosomes and atypical aggregates of normal-sized melanosomes (not shown), similar to those seen in the corresponding mutant mice in vivo (7). All aspects of this abnormal phenotype appear more marked in melan-le than in melan-ep cells, even though le-mutant mice are phenotypically indistinguishable from ep-mutant mice (7, 19-21).

We previously showed that the HPS1 protein is unexpectedly absent in tissues from Hps4-null (le-mutant) mice, as well as from Hps1-null (ep-mutant) mice (7). As shown in Fig. 2, we obtained the same result on western blot analysis of cultured melan-ep and melan-le melanocytes; both lack detectable HPS1 protein. We could not assay HPS4 protein in these cells, as our human HPS4 polyclonal antisera and monoclonal antibodies do not recognize the mouse HPS4 protein. Semi-quantitative RT-PCR showed that the amount of Hps1 mRNA is approximately equal in wild-type melan-a versus melan-le cells (data not shown), indicating that HPS4 protein apparently is not required for transcription and biogenesis of Hps1 mRNA. These results suggest instead that either HPS4 is required for biosynthesis of HPS1, or that absence of HPS4 destabilizes HPS1.

To determine whether HPS4 is required for biosynthesis of HPS1, we over-expressed human HPS4 in Hps1-null melan-ep mouse melanocytes by stable transformation with a human HPS4 cDNA expression plasmid, and human HPS1 in Hps4-null melan-le melanocytes by transformation with a human HPS1 expression plasmid. As expected, melan-ep cells transfected with the HPS1 expression plasmid produced abundant human HPS1 protein (Fig. 2) and were complemented to a normal pigmentation and melanosomal cellular phenotype (Fig. 1).
Likewise, melan-*le* cells transfected with the *HPS4* expression plasmid produced abundant human HPS4 protein (Fig. 2) and were complemented to a normal cellular phenotype (Fig. 1). Moreover, over-expression of human HPS4 protein in transfected melan-*le* cells fully restored expression of endogenous HPS1 protein. In contrast, melan-*ep* cells transfected with the *HPS4* expression plasmid produced abundant human HPS4 protein (Fig. 2), although they of course still expressed no endogenous HPS1 protein (Fig. 2), but showed no apparent correction of the abnormal pigmenitary or melanosomal phenotype (Fig. 1). However, melan-*le* cells transfected with the *HPS1* expression plasmid produced abundant human HPS1 protein and showed partial correction of the abnormal cellular phenotype, presumably due to correction of the secondary HPS1 deficit in these cells. These results show clearly that HPS4 is not required for biosynthesis of HPS1 protein, but that HPS4 instead most likely stabilizes HPS1 as part of a protein complex.

*HPS1 and HPS4 Co-Immunoprecipitate*—To determine whether HPS1 and HPS4 are in a protein complex, we independently immunoprecipitated HPS1 and HPS4 from human MNT1 melanoma cells, and subsequently assayed HPS4 protein in the HPS1 immunoprecipitate and vice versa. As shown in Fig. 3, monoclonal antibody to human HPS1 detected the typical HPS1 doublet in the HPS4 immunoprecipitate. Likewise, polyclonal antibody to human HPS4 detected a single band in the HPS1 immunoprecipitate, corresponding to human HPS4. Thus, HPS1 and HPS4 indeed co-immunoprecipitate, indicating they are in a protein complex. Similarly, we detected interaction between the human HPS4 and mouse HPS1 proteins in vivo, assayed both by co-immunoprecipitation of mHPS1-hHPS4 from melan-le cells transfected by the pIREShyg2-*HPS4* expression plasmid (not shown), and by expressing human His-tagged HPS4 in transfected mouse melan-le melanocytes, purifying the His-tagged HPS4 protein on a metal ion column,
and immunoblotting the bound protein for HPS1 (Fig. 3). Together, these results demonstrate that HPS1 and HPS4 are components of a protein complex in vivo.

_HPS1 and HPS4 do not Interact in a Yeast Two-Hybrid System, but HPS4 Binds to Itself_  
To determine whether HPS1 and HPS4 interact directly, we used a yeast two-hybrid system to test interaction between full-length HPS1 and HPS4. Appropriate combinations of mouse HPS1 and HPS4 binding domain and activation domain constructs were co-transfected into yeast strain AH109 and the co-transformants were plated on SD/-Ade/-His/-Leu/-Trp. As shown in Fig. 4, we saw no growth for any of the HPS1-HPS4- co-transformants in any combination, indicating that mouse HPS1 and HPS4 do not interact directly, at least in this yeast system. However, as shown in Fig. 4, we observed vigorous growth and blue color for the HPS4-HPS4 combination, indicating that HPS4 monomers interact strongly with each other. We observed no apparent HPS1-HPS1 interaction. We obtained identical results using human HPS1 and HPS4 constructs (not shown). These results indicate that HPS1 and HPS4 may not interact directly, but that HPS4 self-associates to at least a dimer.

_HPS1 and HPS4 are Components of a ~500 kDa Cytoplasmic Vesicular-Organellar Complex, BLOC-3—_We previously showed that HPS1 is part of a >500 kDa cytoplasmic vesicular-organellar complex and a ~200 kDa cytosolic complex (24). To determine which of these high molecular weight HPS1 complexes contains HPS4, we re-interrogated the immunoblots used in the previous study, now using antisera to HPS4. As shown in Fig. 5A, HPS1 and HPS4 are both contained in the same >500 kDa cytoplasmic vesicular/organellar fraction from FME melanoma cells. In contrast, the HPS4 protein was undetectable in cytosolic fractions from both FME melanoma cells (Fig. 5B) and human lymphoblastoid cells (not shown).
These results demonstrate that the >500 kDa cytoplasmic vesicular-organellar complex represents BLOC-3.

A Discrete ~200 kDa HPS1-HPS4 Complex, BLOC-4, is a Module of BLOC-3—We previously showed that treatment of the >500 kDa BLOC-3 complex with 100 mM Tris-HCl results in release of HPS1 as a ~200 kDa post-Tris HPS1 complex, which we speculated might be identical to the ~200 kDa HPS1 complex observed in the cytosol (24). To determine whether HPS4 is also released from BLOC-3 by Tris-HCl, we re-interrogated the immunoblots used in the previous study, now using antisera to HPS4. As shown in Fig. 5C, treatment of the FME melanoma vesicular/organellar fraction with 100 mM Tris-HCl results in release of both HPS1 and HPS4 into the same ~200 kDa sub-fractions. Thus, HPS1 and HPS4 are both released from BLOC-3 into a ~200 kDa post-Tris complex.

To determine whether HPS1 and HPS4 released from BLOC-3 by Tris-HCl reside together in a single ~200 kDa post-Tris complex or in two different ~200 kDa post-Tris complexes, we tested whether HPS1 and HPS4 could still be co-immunoprecipitated after dissociation from BLOC-3 by 100 mM Tris-HCl. As shown in Figure 6, treatment of the vesicular/organellar fraction of MNT1 melanoma cells with Tris-HCl did not affect subsequent co-immunoprecipitation of HPS1 and HPS4, indicating that these two proteins are released from BLOC-3 in a single ~200 kDa post-Tris complex that we designate BLOC-4. BLOC-4 thus represents a discrete module of BLOC-3, and is clearly distinct from the ~200 kDa cytosolic HPS1 complex, which does not contain HPS4 (Fig. 5B) and which we tentatively designate as BLOC-5. The fractionation scheme and relationship of BLOC-3, BLOC-4, and BLOC-5 is shown in Fig. 7.
DISCUSSION

HPS is a group of autosomal recessive diseases associated with defective biogenesis of a specific group of organelles: melanosomes, lysosomes, platelet dense granules, and certain other granular elements. This unique constellation of related disorders suggests that there must be specific biosynthetic pathways particular to these organelles, an idea supported by recent cell biological evidence (27). In the mouse, at least 16 different loci are associated with HPS-like phenotypes (9, 10). In humans, six genes have been associated with HPS, each corresponding to one of the mouse HPS loci (4-8). These genetically defined loci provide an avenue to elucidate the physical components required for mammalian organellogenesis.

There has been considerable recent progress in understanding the relationships among these HPS-related genes, and several of the corresponding proteins have now been shown to be associated with each other in poly-protein complexes. Mouse mocha (mh)/human AP3D1 and mouse pearl (pe)/human AP3B1 both encode components of the heterotetrameric AP-3 adaptor complex (28). Mouse pallid (pa), muted (mu), and cappuccino (cno) encode components of a novel “biogenesis of lysosome-related organelles complex”, BLOC-1 (29,30). Very recently, we showed that mouse ruby-eye (ru)/human HPS6 and mouse ruby-eye-2 (ru2)/human HPS5 encode components of another novel complex, BLOC-2 (8). Here, we show that the HPS1 and HPS4 proteins are components of two additional lysosome-related organelar protein complexes, BLOC-3 and BLOC-4 (Fig. 7), and that cytosolic HPS1 appears to be part of yet another complex, BLOC-5.

Absence of HPS4 appears to result in destabilization of HPS1. Similar observations have been made for other HPS-related protein complexes. Steady-state levels of the AP-3 β3A
protein are decreased in mocha (Ap3d1-mutant) as well as in pearl (Ap3b1-mutant) mice (15, 31). Similarly, steady-state levels of the pallidin protein are reduced in both muted and reduced pigmentation (rp)-mutant mice (29). We have shown that the HPS1 protein is absent in light ear (le) as well as pale ear (ep)-mutant mice (7). However, the level of HPS1 protein is not reduced in pearl (pe; Ap3b1-mutant) mice and the levels of the four AP-3 component proteins are not reduced in pale-ear (ep; Hps1-mutant) mice (7), suggesting that HPS1 is not integrally associated with the AP-3 adaptor complex.

BLOC-4 appears to be a discrete HPS1-HPS4-containing module of BLOC-3 that is released by treatment with Tris-HCl. Studies using a yeast two-hybrid system indicate that HPS1 may not interact directly with HPS4, nor with itself, whereas HPS4 does interact with other molecules of HPS4. The relationship between BLOC-3 and BLOC-4, their other components, and the stoichiometry of these components, remains to be determined. In the cytosol HPS1 is contained in another ~200 kDa complex (24) that does not contain HPS4, and which thus appears to be distinct from BLOC-4. We tentatively denote this cytosolic HPS1 complex as BLOC-5.

BLOC-3 is detected in a partially purified cytoplasmic fraction that contains vesicles and organelles. We have previously shown that non-cytosolic HPS1 is principally localized in uncoated small vesicles and early-stage melanosomes (24), and that HPS4 partially co-localizes with HPS1 in vesicles and organelles (7). BLOC-3, and its component BLOC-4, thus appear to play a key roles in trafficking newly-synthesized cargo
proteins to nascent lysosome-related cytoplasmic organelles.

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1 The abbreviations used are: HPS, Hermansky-Pudlak syndrome; MIM, Mendelian Inheritance in Man; BLOC, biogenesis of lysosome-related organelles complex; TPA, phorbol 12-myristate 13-acetate; HRP, horseradish peroxidase.
FIG. 1. Complementation and cross-complementation analysis of wild-type (melan-a), HPS1-null (melan-ep), and HPS4-null (melan-le) mouse melanocytes. Upper panels show untransfected cells. Middle panels show cells stably transformed by pIREShyg2-HPS1, a plasmid expressing full-length human HPS1 HIS-tagged at the amino terminus. Lower panels show cells stably transformed by pIREShyg2-HPS4, a plasmid expressing full-length human HPS4 HIS-tagged at the amino terminus. Each panel shows a representative field of unstained, living cells. Pigmented cytoplasmic granules are melanosomes. Size bars indicate 25 microns. All photographs were taken under identical conditions of illumination and exposure.

FIG. 2. Expression of HPS1 and HPS4 proteins in mouse melanocyte lines and human MNT1 melanoma cells. Cells were immunoblotted using: A, anti-HPS1 monoclonal antibody or B, anti-HPS4 polyclonal antibody. Lanes: 1, human MNT1 melanoma cells; 2, mouse melan-a melanocytes; 3, melan-ep melanocytes; 4, melan-ep melanocytes transformed by pIREShyg2-HPS1; 5, melan-ep transformed pIREShyg2-HPS4; 6, melan-le melanocytes; 7, melan-le transformed pIREShyg2-HPS1; 8, melan-le transformed pIREShyg2-HPS4. Cells were lysed as described and western blotted using either monoclonal antibody to human HPS1 or polyclonal antisera to human HPS4. The human HPS4 protein reproducibly migrates at ~90 kDa on SDS-PAGE, versus its predicted M.W. of 76.9 kDa (7).

FIG. 3. Co-immunoprecipitation and co-purification of the HPS1 and HPS4 proteins from

FIG. 4. Analysis of HPS1 and HPS4 interactions in a yeast two-hybrid system. Yeast strain AH109 was transformed with constructs expressing the indicated proteins and co-transformants were spotted on A, low stringency (-Trp/-Leu/X-alpha Gal; not shown) and B, high stringency (-Trp/-Leu/-His/-Ade/X-alpha Gal) media. Interaction of proteins was assessed by growth and blue color on high stringency plates. Co-transformants pGBK7-53 and pGADT7-T are a positive control for interacting proteins; pGBK7-LAM and pGADT7-T are negative controls. BD=binding domain; AD=activation domain.

FIG. 5. Analysis of the HPS1 and HPS4 proteins in gel filtration fractions of human FME melanoma cells. A 10,000 g cytoplasmic vesicular-organellar fraction and a 100,000 g cytosol fraction were further fractionated by exclusion chromatography on a calibrated Superdex 200
column (7), and column fractions were then analyzed by western blotting using monoclonal antibodies to human HPS1 (upper panels) and HPS4 (lower panels). A, Vesicular-organellar fraction prepared in 25 mM HEPES-KOH, pH 6.8; B, cytosol fraction prepared in 25 mM HEPES-KOH, pH 6.8; C, vesicular-organellar fraction prepared in 100 mM Tris-HCl (pH 7.5). Identical results were obtained on analysis of non-pigmented lymphoblastoid cells. The elution positions of standard proteins are indicated at the top.

FIG. 6. Interaction of HPS1 and HPS4 in BLOC-4 is insensitive to Tris-HCl. The 10,000 g vesicular-organellar fraction was purified from a lysate of human MNT1 melanoma cells and treated with either Na-phosphate buffer or buffer containing 100 mM Tris-HCl, which dissociates HPS1 and HPS4 from BLOC-3. Proteins were then immunoprecipitated using monoclonal antibody to HPS1 (lanes 2, 4) or normal mouse IgG (lanes 1, 3) and immunoprecipitates were then analyzed by western blot using polyclonal antisera to human HPS4. Lanes: 1, Tris-treated, mock immunoprecipitate; 2, Tris-treated, HPS1 immunoprecipitate; 3, no Tris, mock immunoprecipitate; 4, no Tris, HPS1 immunoprecipitate. Similar results were obtained when proteins were immunoprecipitated using polyclonal antibody to HPS4 and detected using monoclonal antibody to HPS1 (data not shown).

FIG. 7. Fractionation schem of the BLOC-3, BLOC-4, and BLOC-5 complexes. Melanoma cell homogenate is centrifuged at 1,000 g to yield a post-nuclear supernatant that is further centrifuged at 10,000 g to yield a fraction containing large vesicles and organelles, and BLOC-3 (>500 kDa; HPS1 + HPS4). Treatment of the vesicular-organellar fraction with 100 mM Tris-
HCl yields BLOC-4 (~200 kDa; HPS1 + HPS4). Centrifugation of the post-nuclear supernatant at 10,000 g, and subsequently 100,000 g, yields a cytosol fraction that contains BLOC-5 (~200 kDa; HPS1, no HPS4).
melan-a  melan-ep  melan-le

+ HPS1

+ HPS4
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