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An immunoblotting assay to facilitate the molecular diagnosis of Hermansky-Pudlak syndrome

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Abstract

Hermansky-Pudlak syndrome (HPS) comprises a constellation of human autosomal recessive disorders characterized by albinism and platelet storage pool deficiency. At least eight types of HPS have been defined based on the identity of the mutated gene. These genes encode components of four ubiquitously expressed protein complexes, named Adaptor Protein (AP)-3 and Biogenesis of Lysosome-related Organelles Complex (BLOC)-1 through -3. In patients of Puerto Rican origin, the molecular diagnosis can be based on analysis of two founder mutations. On the other hand, identification of the HPS type in other patients relies on the sequencing of all candidate genes. In this work, we have developed a biochemical assay to minimize the number of candidate genes to be sequenced per patient. The assay consists of immunoblotting analysis of extracts prepared from skin fibroblasts, using antibodies to one subunit per protein complex. The assay allowed us to determine which complex was defective in each of a group of HPS patients with unknown genetic lesions, thus subsequent sequencing was limited to genes encoding the corresponding subunits. Because no mutations within the two genes encoding BLOC-3 subunits could be found in two patients displaying reduced BLOC-3 levels, the possible existence of additional subunits was considered. Through size-exclusion chromatography and sedimentation velocity analysis, the native molecular mass of BLOC-3 was estimated to be 140 ± 30 kDa, a value most consistent with the idea that BLOC-3 is a HPS1•HPS4 heterodimer (~156 kDa) albeit not inconsistent with the putative existence of a relatively small third subunit.

Keywords

AP-3; BLOC-1; BLOC-2; BLOC-3; Hermansky-Pudlak syndrome; Immunoblotting; Molecular Diagnosis

Introduction

The term Hermansky-Pudlak syndrome (HPS) is used to define a group of at least eight human genetic disorders (HPS-1 through -8) characterized by oculocutaneous albinism and storage

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pool deficiency due to defects in the formation of melanosomes and platelet dense granules (δ-granules), respectively [1,2]. Although these two manifestations of HPS are shared with Chediak-Higashi syndrome, the differential diagnosis between these two syndromes relies on the occurrence in the latter of severe innate immunodeficiency, often leading to an accelerated lymphoproliferative phase, and characteristic giant intracellular organelles [3,4]. Like other forms of albinism, HPS is associated with reduced visual acuity and nystagmus [1-3]. The storage pool deficiency leads to prolonged bleeding times and easy bruising [1-3], and only on rare occasions to bleeding events requiring blood or platelet transfusion [5]. Currently, the clinical diagnosis of HPS is based on the manifestations of oculocutaneous albinism and the apparent absence of dense granules in peripheral blood platelets - as visualized by electron microscopy using the whole-mount technique [6]. HPS has been described in patients from several different ethnicities, and is considered a rare disorder worldwide but relatively common in the island of Puerto Rico, owing to the occurrence of two independent founder effects [1, 7,8].

At the molecular level, the eight known types of autosomal recessive HPS are defined on the basis of the identity of the mutated gene (Table 1). The most prevalent type of HPS is HPS-1, which is due to mutations in a gene first identified through positional cloning of the genetic lesion shared by HPS patients from Northwestern Puerto Rico [7]. Other HPS genes were subsequently identified through positional cloning and/or candidate gene approaches [8-13]. Two characteristics are shared by all of these genes: (i) their expression in a wide variety of tissues and cell types, despite the main manifestations of HPS being a consequence of defects in cell-type-specific organelles, and (ii) the association of their products into one of four stable protein complexes, namely Adaptor Protein (AP)-3 (containing the product of the gene mutated in HPS-2), Biogenesis of Lysosome-related Organelles Complex (BLOC)-1 (containing the products of the genes mutated in HPS-7 and -8), BLOC-2 (containing the products of the genes mutated in HPS-3, -5 and -6) and BLOC-3 (containing the products of the genes mutated in HPS-1 and -4) (Table 1) [1].

In a few HPS cases in which the molecular consequences of disease-causing mutations have been studied, the mutations were found not only to affect the protein encoded by the mutant gene but also to lead to secondary destabilization and degradation of the corresponding protein complex [9]; a similar phenomenon was observed in mouse strains carrying mutations in the orthologs of each of the genes associated with HPS in humans [10,12,14-17]. This has led to the notion that the different types of HPS could be classified into clinically relevant groups depending upon the affected protein complex [1]. Accordingly, the manifestations of HPS-1 and -4 would result from a common deficiency in BLOC-3 function, those of HPS-2 from defects in AP-3, those of HPS-3, -5 and -6 would result from BLOC-2 deficiency, and those of HPS-7 and -8 from defects in BLOC-1. Clinical characterization of some HPS types has begun to lend support to this notion. Thus, both HPS-1 and -4, and not other HPS types, are associated with increased risks of developing pulmonary fibrosis (which can be fatal) and gastro-intestinal (GI) manifestations such as granulomatous colitis [18-23], although that may not be the case for HPS-1 patients from a Swiss isolate [3]. HPS-2, and not the other HPS types, is associated with recurrent infections due to chronic neutropenia and other deficiencies in the innate immune system [24-28]. In contrast, HPS-3, -5 and -6 have been clinically characterized as a mild form of HPS [8,11,29-32]. Taken together, these observations underscore the potential significance of identifying the disease type - or at least the deficient protein complex - for prognosis and, in the future, for consideration of eventual treatment options.

Because of the occurrence of two founder effects in Puerto Rico, the molecular diagnosis of HPS patients of this ethnic background can be based on simple polymerase chain reaction (PCR) assays [7,8,33]. By analogy, focused mutation analyses could also be undertaken for HPS patients from other genetic isolates [32]. Nevertheless, the molecular diagnosis (and

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determination of disease type) of HPS patients of non-Puerto Rican origin is largely based on sequencing of all coding regions and intron-exon boundaries of every single candidate gene. Exactly which genes should be considered candidates for mutation analyses in HPS patients is dependent upon different criteria. At the very least, the eight genes already associated with the human disease (*i.e., HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DTNBP1* and *BLOC1S3*) should be included. It can also be argued that other genes encoding subunits of AP-3 or a BLOC should be taken into consideration as well, especially those for which mutations in the mouse counterpart have been shown to result in HPS-like phenotypes [34-37], thus potentially raising the number of candidate genes to eighteen (Table 1). On the other hand, one might consider excluding *AP3S1* and *AP3S2* from the list on the basis of an argument of genetic redundancy (*i.e.*, both genes encode alternative AP-3 subunits with apparently the same molecular function [38]) or *AP3D1* on the basis of the occurrence in *Ap3d1*-null mice of neurological phenotypes not yet observed in HPS patients [34]. Finally, one might choose to expand the list even further by including the genes encoding subunits of the Homotypic vacuolar Protein Sorting complex (HOPS), given reported mutations within the gene encoding its VPS33A subunit in the buff mouse model [39], or the *RAB38* gene, given a reported mutation within its ortholog in rat models of HPS [40]. In any event, the number of candidate genes to be sequenced for each non-Puerto Rican patient with a new diagnosis of HPS is large enough to be regarded as a challenging task by most molecular diagnosis laboratories.

In this paper, we describe an immunoblotting-based assay that we have developed with the aim of minimizing the number of candidate genes to be sequenced for each new HPS patient. The goal of the assay is to determine which of the four protein complexes so far associated with HPS in humans (*i.e.*, AP-3 and BLOC-1, -2 and -3) may be expressed at significantly reduced steady-state levels in the patient's cells, such that subsequent mutation screenings can be restricted to only those genes encoding subunits of the defective complex. As a proof of principle, we have examined cell extracts from: (*i*) HPS patients of known genetic lesions, and (*ii*) patients for whom preliminary sequencing efforts had yielded inconclusive results.

Materials and methods

Antibodies

The following antibodies raised in our laboratory were used for the immunoblotting assay: mouse monoclonal antibody (mAb) 2G5 against the pallidin subunit of human BLOC-1 (developed as previously described for mAb 2G6 against the same protein; [41]), affinity-purified rabbit polyclonal antibody HP6d against the HPS6 subunit of human BLOC-2 [42], and mouse mAb 1F7A8 against the HPS4 subunit of human BLOC-3 [43]. The commercial antibodies were: mouse mAb against the μ3A subunit of AP-3 (anti-p47A), which was purchased from BD Transduction Laboratories (Lexington, KY), and mouse mAbs against α-tubulin (clone DM1A) and β-actin (clone AC15), which were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies used in preliminary experiments were against the σ3A and β3A subunits of AP-3 [38,44], the dysbindin subunit of BLOC-1 [17] and the HPS3 subunit of BLOC-2 [42,45]. Horseradish peroxidase (HRP)-conjugated antibodies against mouse or rabbit IgG were from GE Healthcare (Waukesha, WI).

Patients

All procedures involving human subjects were performed according to the Declaration of Helsinki Principles. All patients were enrolled in a protocol approved by the Institutional Review Boards of the National Institute of Child Health and Human Development and the National Human Genome Research Institute of the National Institutes of Health (NIH, Bethesda, MD) for the study of clinical and molecular aspects of HPS. Written informed consent was obtained from the patients or, if appropriate, their parents. Patient numbers

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correspond to a master file of all patients enrolled in this protocol. The diagnosis was based on the occurrence of oculocutaneous albinism and the apparent absence of platelet dense granules as visualized by whole-mount electron microscopy [6]. Two patients, referred herein to as "43*" and "47*," were tentatively diagnosed as suffering from "HPS-related" disease. These two patients presented with albinism (and its associated visual defects) and GI manifestations reminiscent of those observed in HPS types 1 and 4; patient 47* also manifested with bleeding diathesis. However, dense granules were detected in the platelets from both patients 43* and 47*. Patient 36 was previously shown to carry compound heterozygous nonsense mutations in the *HPS4* gene [20] and is herein referred to as "HPS-4 control." Fibroblasts were obtained from small skin biopsies and cultured as described [30]. Frozen cell pellets with no identifier other than patient numbers were shipped by express mail to Los Angeles, CA, for subsequent extract preparation and immunoblotting (see below) according to a protocol approved by the Institutional Review Board of the University of California, Los Angeles.

Cell culture

Primary cultures of skin fibroblasts derived from apparently healthy donors (GM00037 and GM03651) and from patients diagnosed with HPS-1 (GM14609) and HPS-2 (GM17890), as well as Epstein-Barr virus (EBV)-transformed B-lymphoblastoid lines derived from an apparently healthy donor (AG10111) and from patients diagnosed with HPS-1 (GM14606 and GM13958) and HPS-6 (GM17881), were all obtained from Coriell Cell Repositories (Camden, NJ). Human HeLa and MNT-1 cells were obtained and cultured as described elsewhere [46]. Primary fibroblasts were grown on monolayers in plastic flasks containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. EBV-transformed B-lymphocytes were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 15% (v/v) fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin.

Cell extract preparation

Whole-cell detergent extracts were prepared using lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, 1 mM NaF, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin and 1 μg/ml pepstatin A. Cells were suspended in lysis buffer, incubated on ice for 45 minutes, and then sonicated for 5 seconds using a Branson 450 sonifier (Branson Ultrasonic Corporation, Danbury, CT) equipped with a microtip. The resulting lysate was cleared by centrifugation at 15,000 × *g* for 10 minutes at 4°C. Total protein concentration in each extract was estimated using the Protein Assay reagent (Bio-Rad, Richmond, CA) and referred to a standard prepared using crystallized, fatty-acid-free, bovine serum albumin (Sigma-Aldrich). Following normalization of the total protein concentration by dilution with appropriate volumes of lysis buffer, an equal volume of gel sample buffer (0.1 M Tris-HCl, pH 6.8, 24%, w/v, glycerol, 8%, w/v, SDS, 0.2 M dithiothreitol, and 0.1%, w/v, bromophenol blue) was added to each extract prior to heating at 95°C for 5 minutes.

Immunoblotting

Cell extracts prepared as described above (typically 10 μg total protein per sample) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system [47] on commercial pre-cast 4-20% (T) gradient gels (Invitrogen, Carlsbad, CA). Rainbow™ colored molecular mass markers (GE Healthcare) were run in parallel. Fractionated proteins were electro-transferred from the gels onto polyvinylidene disulfluoride membranes (BioRad) for 90 min at 100 V in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20%, v/v, methanol) at about 4°C. The membranes were blocked overnight at 4°C in blocking buffer

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(5%, w/v, non-fat milk and 0.2%, w/v, Tween 20 in PBS) and incubated for 1 h at room temperature with the primary antibody diluted in blocking buffer. The primary antibody working dilutions were: purified rabbit polyclonal HP6d against HPS6, 1:400; ascites fluid containing mAb against α-tubulin, 1:10,000; purified mAb against μ3A, 1:250; ascites fluid containing mAb 2G5 against pallidin, 1:500; and ascites fluid containing mAb 1F7A8 against HPS4, 1:500. Following incubation with the primary antibody, membranes were washed four times with phosphate-buffered saline (PBS) and then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG; GE Healthcare) diluted 1:6000 in blocking buffer. Following additional four washes with PBS, bound antibodies were detected using ECL Plus™ Western Blotting Detection Reagent (GE Healthcare) - except for membranes incubated with antibody against α-tubulin or β-actin, for which the ECL™ reagent was used - and then exposing the membranes to X-ray films. In order to re-probe membranes with different primary antibodies, membranes were first incubated in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) for 10 min at 37° C, washed three times with PBS, and then incubated in blocking buffer for a minimum of 2 h at room temperature.

Mutation analysis

Genomic DNA was extracted from the patients' blood peripheral leukocytes using standard protocols [48]. Primer pairs were designed for PCR amplification of each exon and their adjacent intron sequences of HPS human candidate genes (Table 1). Primer sequences are available upon request and have been previously described for *HPS1* [49], *AP3B1* [25], *HPS3* [29], *HPS4* [20], *HPS5* [30] and *HPS6* [32]. Standard PCR amplification procedures [48] were employed. Direct sequencing of PCR products was performed using Beckman CEQ2000 (Beckman Coulter, Fullerton, CA) or ABI3100 (Applied Biosystems, Foster City, CA) automated sequencers, following the manufacturers' protocols. Cloning of PCR products for sequencing of exon 17 of each *HPS1* allele from patient 128 was performed using the Zero Blunt TOPO PCR Cloning Kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Estimation of the native molecular mass of BLOC-3

Size-exclusion chromatography and sedimentation velocity analysis of freshly prepared cytosol from human HeLa cells were carried out as previously described [42]. Cytosol was prepared from human MNT-1 cells as described for the preparation of HeLa cytosol [42] except for the use of a different homogenization buffer, which consisted of 0.3 M Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin and 1 μg/ml pepstatin A. Size-exclusion chromatography of MNT-1 cytosol (0.2 ml, 1.3 mg total protein) on a Superose 6 column (1 5 60 cm, GE Healthcare) equilibrated with homogenization buffer, and fractionation by ultracentrifugation on a linear 5-20% (w/v) sucrose gradient in the same buffer, were performed as described [50]. In all cases, the presence of BLOC-3 in the resulting fractions was detected by immunoblotting using the mAb 1F7A8 against the HPS4 subunit. The estimated Stokes radius (from the size-exclusion chromatography) and sedimentation coefficient (from the sedimentation velocity analysis) were used to calculate the molecular mass and frictional ratio of native BLOC-3, assuming a partial specific volume in the normal range of 0.72-0.75 cm^3/g for soluble proteins [51]

Results and discussion

Specificity and sensitivity of antibodies to representative subunits of AP-3 and the BLOCs

We first sought to determine which of the antibodies available to us and capable of recognizing human AP-3 and BLOC subunits would be suitable for the immunoblotting assay. Although

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we had previously raised a number of rabbit polyclonal antibodies against almost all BLOC subunits [17,42,45,46,50], preference was given to mAbs since they can be obtained from propagative sources (*i.e.*, hybridomas).

In the case of BLOC-1, we have recently raised mAbs to its pallidin subunit by using the recombinant human protein as the immunogen [41]. One mAb, produced by hybridoma 2G6, was able to recognize the pallidin protein from various mammalian species, and its specificity was evidenced by the lack of cross-reacting bands in extracts prepared from the kidney of pallid mutant mice (Fig. 1A, *right panel*), which carry a nonsense mutation in the pallidin-encoding gene [35]. Another mAb, produced by hybridoma 2G5, reacted only with the human protein (Fig. 1A, *left panel*). Although both mAbs are suitable for the assay, in this work we chose to use the species-specific mAb 2G5. We analyzed extracts prepared from primary skin fibroblasts and EBV-transformed B-lymphocytes, which were obtained from a public cell repository and included samples from HPS patients of known genetic lesions (Fig. 1B), to determine the sensitivity of detection of endogenous pallidin by this mAb. As shown in Fig. 1C (*upper left panel*), pallidin was readily detected in these samples as a single protein band with a mobility corresponding to a protein of ~24 kDa (a value consistent with previous reports; [35,50]). Under these experimental conditions, the protein could be detected in as little as ~3 μg of total protein from fibroblasts, with reasonable sample-to-sample variability (Table 2).

Owing to the lack of mAbs against human BLOC-2 subunits, we tested two affinity-purified, rabbit polyclonal antibodies: HP3c against HPS3 [45] and HP6d against HPS6 [42]. The latter was chosen as it was found to detect the endogenous cognate antigen (apparent molecular mass of ~90 kDa as in a previous report [42]) with high specificity and sensitivity (Fig. 1C, *lower left panel*, and Table 2). In particular, the fact that the major ~90-kDa protein band recognized by the HP6d antibody was absent from B-lymphocytes of a patient suffering from HPS-6 due to a homozygous frameshift deletion (*HPS6* c.1714-1717delCTGT; Ref. [11]) corroborated its identity as endogenous HPS6 (Fig. 1C, *lower left panel*, lane 8).

In the case of BLOC-3, we have recently generated a mAb against the human HPS4 subunit [43]. As shown in Fig. 1C (*upper right panel*), the mAb allowed detection of endogenous HPS4 from both primary fibroblasts and transformed B-lymphocytes as a protein band of apparent molecular mass ~100 kDa, a value consistent with previous reports [45,52]. Other protein bands of lower apparent masses were also detected and may represent non-specific cross-reactivity. Importantly, the ~100-kDa protein was significantly reduced in cell extracts from patients suffering from HPS-1 due to mutations in the *HPS1* gene encoding the other known BLOC-3 subunit (Fig. 1C, *upper right panel*, lanes 3, 6 and 7). Compared to the antibodies selected for analysis of BLOC-1 and -2, this mAb allowed detection of endogenous HPS4 with lower sensitivity and higher sample-to-sample variability (Table 2).

Finally, in order to detect an AP-3 subunit we tested various antibodies and chose a commercially available mAb against the μ3A subunit. As expected, the mAb recognized its cognate antigen as a ~47-kDa protein band that was virtually absent from the extract prepared from fibroblasts of a HPS-2 patient (Fig. 1C, *lower right panel*, lane 4), who carried compound heterozygous nonsense mutations in the *AP3B1* gene encoding the β3A subunit of AP-3 (p.R509X and E659X; Ref. [25]). Sensitivity of detection of endogenous μ3A was relatively high, although the sample-to-sample variability was not insignificant (Table 2).

Consequently, the above results led us to adopt the following steps for the assay:

1. Cell extracts are prepared and normalized to an equal total protein concentration,
2. Proteins in the extracts (10 μg total protein) are fractionated by SDS-PAGE and transferred to membranes; the membranes are then cut into three pieces along the

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positions corresponding to molecular masses of ~67 and ~30 kDa (based on colored molecular mass markers),

3. The membrane pieces corresponding to low (<30 kDa), intermediate (30-67 kDa) and large (>67 kDa) molecular masses are probed with the antibodies against pallidin (for BLOC-1), μ3A (for AP-3) and HPS4 (for BLOC-3), respectively,
4. The membrane pieces corresponding to intermediate (30-67 kDa) and large (>97 kDa) molecular masses are treated to remove bound antibodies and subsequently re-probed with the antibodies against α-tubulin (for loading control) and HPS6 (for BLOC-2), respectively.

Although the experiments described below were carried out on primary skin fibroblast cultures derived from the patients, in principle the assay should be compatible with the analysis of other cell types (*e.g.*, EBV-transformed B-lymphocytes, peripheral blood leukocytes). It should be mentioned, however, that so far our attempts to run the assay using isolated peripheral blood platelets have been unsuccessful; this was in part due to poor reproducibility in the recovery of extracted proteins (data not shown). Finally, taking into account the sample-to-sample variability resulting from detection of these complex subunits in cells from apparently healthy individuals (Table 2) and the expectation that steady-state levels of multi-subunit protein complexes may not necessarily correlate with gene dosage of individual subunits, no attempts were made to use this assay to detect carrier state.

Analysis of samples from HPS patients of unknown genetic lesions

Even when the results described above provided a first proof-of-principle that the assay can allow identification of defective protein complexes in HPS patients, we sought to assess its potential to assist in the molecular diagnosis of potentially “hard-to-solve” cases. To this end, we chose to analyze fibroblast cell samples derived from a subset of patients of non-Puerto Rican origin and unknown genetic lesion. Five patients (numbers 45, 88, 94, 128 and 156) had received unequivocal diagnosis of HPS, while another two (patients 43* and 47*) had been tentatively diagnosed as suffering from “HPS-like” disease owing to the occurrence of platelet dense granules as detected by electron microscopy. Patient 156 was new in the HPS protocol at the time that these experiments were performed, and no prior sequencing analysis had been carried out. For all the other patients, preliminary sequencing efforts had failed to identify the disease-causing mutations (data not shown).

Frozen fibroblast cell pellets from each of these patients, as well as from an apparently normal individual and a patient with the molecular diagnosis of HPS-4, were analyzed using the assay as described in the previous section. Results representative of three assays are shown in Fig. 2. While none of the patients’ cells displayed dramatic reductions in the steady-state levels of μ3A (AP-3) or pallidin (BLOC-1), two of them (patients 88 and 156) expressed significantly decreased levels of HPS6 (BLOC-2) and three (patients 45, 94 and 128) besides the control HPS-4 patient’s cells expressed little or no detectable HPS4 (BLOC-3). Notably, the only two patients for whom we could not detect a defect in any of the four protein complexes were patients 43* and 47*; as mentioned above, these two patients likely belong a clinical entity different from *bona fide* HPS. Therefore, these results suggested that patients 88 and 156 suffer from HPS due to mutations causing BLOC-2 deficiency, and that patients 45, 94 and 128 suffer from HPS due to BLOC-3 deficiency. Based on reported differences in the severity of HPS disease caused by BLOC-2 deficiency (in HPS-3, -5 or -6 [8,11,29-32]) or BLOC-3 deficiency (in HPS-1 or -4 [18-23]), one would then expect that patients 88 and 156 should suffer from a mild form of HPS and that patients 45, 94 and 128 should be at a higher risk of developing pulmonary fibrosis or GI disease. Unfortunately, only two of these patients (88 and 45) were examined at an age in which the validity of these predictions could begin to be evaluated: at the time of their last evaluation at the NIH Clinical Center, patients 88 (age 40 years), 94 (age

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2 years), 128 (age 2 years) and 156 (age 19 years) had no signs of interstitial lung disease of GI disease, while patient 45 (age 35 years) presented with a history of GI disease but no interstitial lung disease.

Based on the results from the assay, subsequent mutational analyses were performed for patients 88 and 156 with a focus on only the three genes known to encode BLOC-2 subunits (*i.e., HPS3, HPS5* and *HPS6*) and for patients 45, 94 and 128 with a focus on the *HPS1* and *HPS4* genes known to encode subunits of BLOC-3. In all cases, all exons plus exon-intron boundaries were isolated and sequenced, and in most cases expression of mRNAs was verified by reverse transcriptase-PCR. Technical issues prevented us from sequencing promoter or intronic regions.

In the case of patient 88, no relevant sequence variations were found in *HPS3* or *HPS6*. On the other hand, a missense mutation was found in one of the two copies of the *HPS5* gene. As shown in the electrophoregrams of Fig. 3A, the patient was heterozygous for a single nucleotide change on exon 5 of *HPS5* [c.434G>A] leading to substitution of glutamate for glycine at amino acid position 145 [p.G145E]. Although no mutations were found so far in the other copy of the *HPS5* gene, the possibility that the observed amino acid substitution contributes to the pathogenesis of HPS in this patient is supported by the following: (i) no such substitution was observed upon sequencing of *HPS5* in ~80 chromosomes from individuals of similar ethnic background, (ii) no such variation was found upon searching the human expressed sequence tags (EST) or dbSNP Build 127 databases, and (iii) phylogenetic sequence analyses revealed that glycine at position 145 of human HPS5 is absolutely conserved in HPS5 orthologs from both vertebrates and invertebrates (Fig. 4A). Nevertheless, further analyses of the other copy of *HPS5* in patient 88 will be required before a tentative diagnosis of HPS-5 can be verified.

In the case of patient 156 (*i.e.*, the other patient with BLOC-2 deficiency), sequencing analyses revealed the presence of a homozygous nucleotide change in *HPS3* [c.1189C>T] that leads to substitution of tryptophan for arginine at position 397 [p.R397W] (Fig. 3B). The same allelic variant had been previously described as a disease-causing mutation in other HPS patients [29]. Consequently, patient 156 was diagnosed as suffering from HPS-3 disease. We note that no prior sequencing analysis had been carried out for this patient prior to the immunoblotting assay; hence, in this case the assay was helpful to focus our efforts on just three candidate genes.

In the cases of patients 45, 94 and 128, sequencing analyses were focused on the *HPS1* and *HPS4* genes encoding BLOC-3 subunits. Whereas no mutations were found in either gene for patients 45 and 94 (data not shown), patient 128 was found to be heterozygous for two nucleotide changes within exon 17 of *HPS1*, namely [c.1639G>T] and [c.1645C>T] leading to substitutions of leucine for valine at position 547 and of cysteine for arginine at position 549, respectively (Fig. 3C). We considered the possibility of these substitutions residing on different copies of the *HPS1* gene (*i.e.*, compound heterozygosity). However, isolation of each *HPS1* allele by cloning and subsequent sequencing revealed that both sequence variations are on the same allele (data not shown). No other sequence variations in *HPS1* (or in *HPS4*) were found in this patient. Nevertheless, the following lines of evidence support the notion that one or both of these amino acid substitutions may contribute to the pathogenesis of HPS in patient 128: (i) none of these variants was found upon sequencing of *HPS1* in over 130 chromosomes from individuals of similar ethnic background, (ii) none of these variants was found upon searching the human EST or dbSNP Build 127 databases, and (iii) sequence analyses revealed that valine at position 547 of human HPS1 is conserved in orthologs from most vertebrates - except for the HPS1 ortholog in the zebrafish - and that arginine at position 549 of human HPS1 is absolutely conserved in orthologs from both vertebrates and invertebrates (Fig. 4B).

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Further analyses of *HPS1* in this patient will be required before a diagnosis of HPS-1 can be corroborated.

Does human BLOC-3 contain additional subunits?

Our failure to detect mutations within any of the two genes known to encode BLOC-3 subunits in two HPS patients expressing reduced steady-state levels of this protein complex (patients 45 and 94; Fig. 2) could imply that these patients may bear mutations within the promoter, enhancer or intronic regions of these two genes or, alternatively, that they might carry mutations in a different gene encoding a putative third subunit of BLOC-3. The second alternative was considered given previous estimates of the native molecular mass of the complex, which ranged from ~175 kDa [52] to ~200 kDa [15,53] in non-pigmented cells and from ~200 to over 500 kDa in melanin-producing melanoma cells [15,53]. Because the calculated molecular mass of a HPS1•HPS4 heterodimer approximates 156 kDa, the existence of one ore more additional BLOC-3 subunits was plausible. To re-examine the issue, we fractionated cytosolic proteins extracted from a non-pigmented (HeLa) and a melanin-producing (MNT-1) human cell lines by both size-exclusion chromatography and ultracentrifugation on a linear density gradient. The resulting fractions were analyzed by immunoblotting using our mAb against HPS4 to determine the position of elution of native BLOC-3 from the column chromatography (Fig. 5A) as well as its position following sedimentation through the density gradient (Fig. 5B). The results from size-exclusion chromatography were used to estimate Stoke's radii within the ranges of 48-56 Å and 52-57 Å for endogenous BLOC-3 from HeLa and MNT-1 cells, respectively. In addition, the sedimentation velocity analyses (Fig. 5B) allowed us to estimate sedimentation coefficients within the ranges of 5.9-6.8 S and 5.3-6.6 S for BLOC-3 from HeLa and MNT-1 cells, respectively. These sedimentation coefficient values were in close agreement with those previously reported for BLOC-3 [45,46,52]. Owing to the extent of overlap in these value ranges, we suspect that the molecular composition of BLOC-3 may be identical in both pigmented and non-pigmented cells. In fact, using the above parameters we calculated essentially the same range of molecular masses for BLOC-3 from both cell types, namely 140 ± 30 kDa. The calculated frictional ratios (f/f_o) seemed somewhat different, with mean values of ~1.5 and ~1.6 for BLOC-3 from HeLa and MNT-1 cells, respectively, although statistically these differences were not significant. In any event, the fact that the frictional ratio of BLOC-3 was larger than that expected for a spherical molecule ($f/f_0 = 1$) was consistent with a previous analysis of epitope-tagged forms of the complex [52] and provided a satisfactory explanation for why the molecular mass values calculated by a combination of size-exclusion chromatography and sedimentation velocity analysis (this work and Ref. [52]) were lower than those estimated using only the first method [15,53]. A native molecular mass of 140 ± 30 kDa for BLOC-3 is, consequently, most consistent with the idea that the complex exists as a HPS1•HPS4 heterodimer, although still the putative existence of a relatively small (<15 kDa) third subunit cannot be excluded at this point.

Conclusions

The immunoblotting assay described in this paper can be very useful to minimize the number of candidate genes to be sequenced for each new HPS patient, especially for those of non-Puerto Rican origin. In turn, by focusing on few genes per patient one can pay more attention to sequence variants that could be potentially overlooked, or consider extending the sequencing efforts to include promoter, enhancer and intronic regions.

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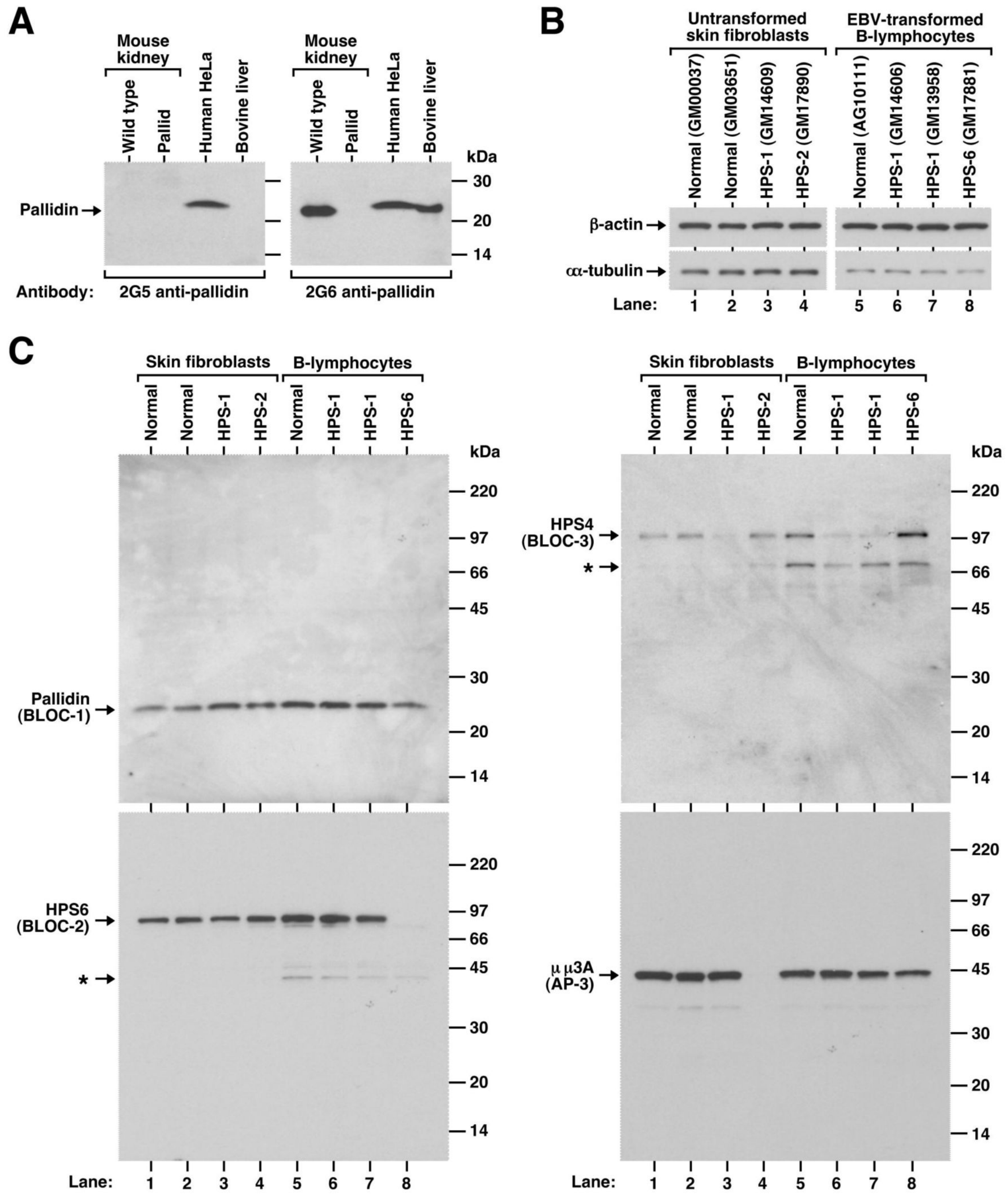

Fig. 1.

Specificity of antibodies selected for the assay. (A) Detergent extracts prepared from kidneys of mice from a wild-type strain (C57BL/6J) and the pallidin-null mutant strain pallid (B6.Cg-*Pldn*^{pa}/J), a whole-cell extract prepared from human HeLa cells, and bovine liver cytosol, were analyzed by immunoblotting using two monoclonal antibodies (mAbs; 2G5 and 2G6) raised against the pallidin subunit of human BLOC-1. Notice that moAb 2G6 recognized the pallidin protein from human, murine and bovine samples, whereas moAb 2G5 recognized only the human protein. (B and C) Detergent extracts prepared from human skin fibroblasts and Epstein-Barr virus (EBV)-transformed B-lymphocytes were analyzed by immunoblotting using antibodies to β-actin and α-tubulin as loading controls (B) and to the pallidin subunit of

BLOC-1, the HPS6 subunit of BLOC-2, the HPS4 subunit of BLOC-3, and the μ3A subunit of AP-3 (C). The cell samples were obtained from Coriell Cell Repository (repository numbers indicated between parentheses) and derived from apparently normal individuals as well as from patients suffering from HPS due to mutations in genes encoding subunits of AP-3 (HPS-2 disease), BLOC-2 (HPS-6 disease) and BLOC-3 (HPS-1 disease). *Asterisks* denote bands deemed to represent non-specific cross-reactivity.
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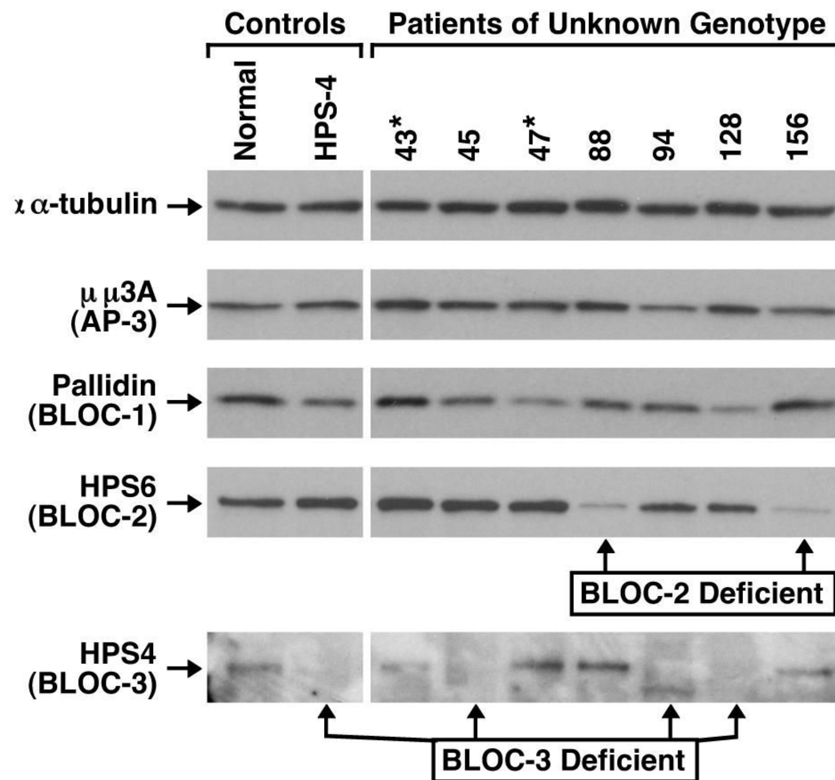

Fig. 2.

Immunoblotting analysis of cell samples derived from patients of unknown genetic lesion. Detergent extracts were prepared from the cultured skin fibroblasts of five patients with clinical diagnosis of HPS (45, 88, 94, 128 and 156), and two patients suffering from HPS-related disease (43* and 47*). As controls, detergent extracts prepared from an apparently normal individual and a patient suffering from HPS-4 (due to known mutations in the HPS4 subunit of BLOC-3) were analyzed in parallel.

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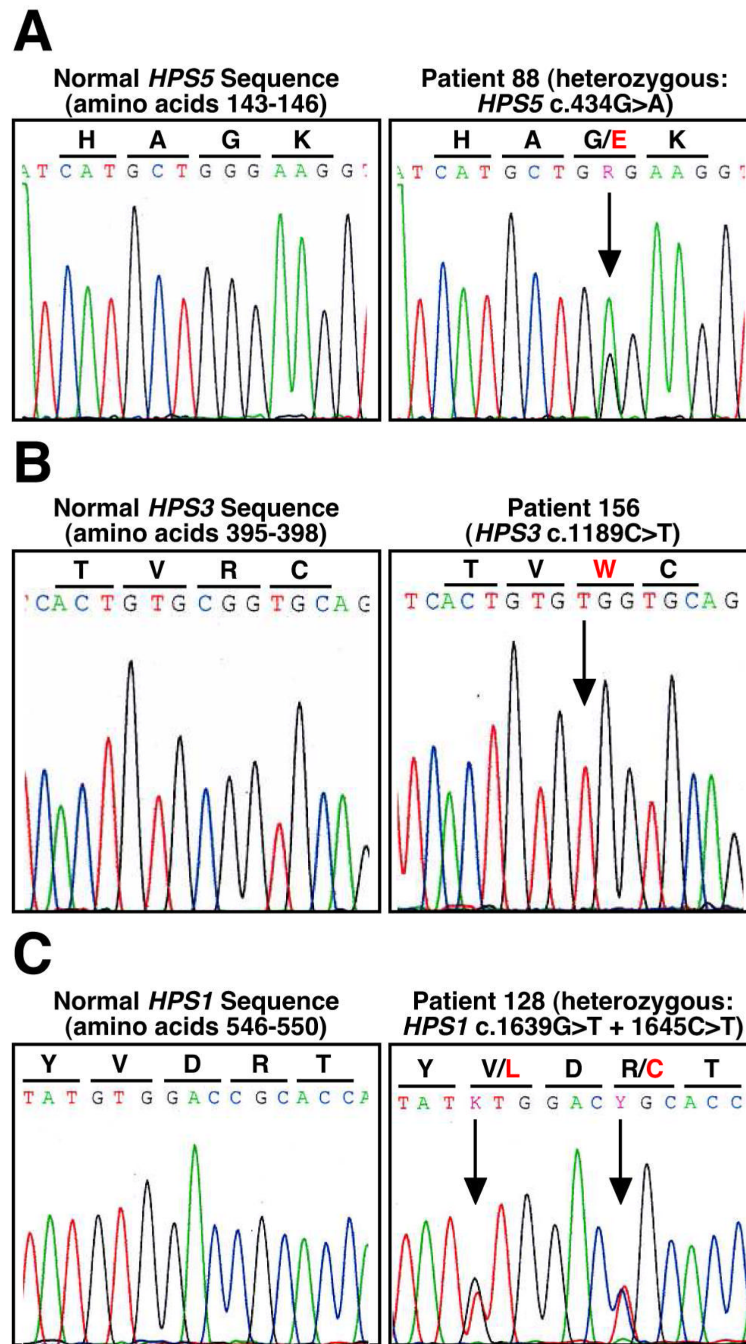

Fig. 3.

Selected portions of electrophoregrams obtained during sequencing of DNA samples from apparently normal individuals (A-C, *left panels*) and HPS patients 88 (A, *right*), 156 (B, *right*) and 128 (C, *right*). See the text for further details.

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A **HPS5 Protein**

	139–152
Human	FVGDHAGKVSAIKL
Chimpanzee	FVGDHAGKVSAIKL
Horse	FVGDHMGKVSAIKL
Pig	FVGDHMGKVSAIKL
Cattle	FVGDHLGKVSAIKL
Dog	FVGDHMGKVSAIRL
Mouse	FVGDHVGKVSAIKL
Chicken	FVGDHVGKVSAIKI
Frog	FVGDHLGKVTAIKL
Zebrafish	FAGDMGGKVSCVRA
Fugu fish	YVGDSGGKVSLLRA
Tetraodon	FVGDSAGKVSCLRA
Fruit fly	YYGDSRGQVSLVLL
Beetle	YCGDNTGRVSVVAL

B **HPS1 Protein**

	536–557
Human	EDFPGLVHFIYVDRTTGQMVAP
Chimpanzee	EDFPGLVHFIYVDRTTGQMVAP
Orangutan	EDFPGLVHFIYVDRTTGQMVAP
Macaque	EDFPGLVHFIYVDRTTGQMVAP
Pig	EDFPGLVHFIYVDRTTGQMVAP
Horse	EDFPGLVHFIYVDRTTGQMVAP
Dog	EDFPGLVHFIYVDRTTGQMVAP
Cattle	EDFPGLVHFIYVDRTTGQMVAP
Rat	EDFPGLVHFIYVDRTTGQMVAP
Mouse	EDFPGLVHFIYVDRTTGQMVAP
Opossum	EDFPGLVHFIYVDRMTGQMVAP
Platypus	EDFPGLVHFIYVDRLGGQMVAP
Chicken	EDFPGLVHFIYVDRTAGQMMAP
Frog	EDFPGLIHFIYVDRTVGQMVAP
Zebrafish	EEFPGLIHFIYMDRSSGQMIAP
Tetraodon	PDFPGLIHFICVDRSTGQMIAP
Beetle	QEFPGLVHFLYIDRVNHRVTAP
Fruit fly	EDFPGLVHFMYVNRSRGQMLAP
Honey bee	EEFPGLVHFIYIDRITHRLTAP

Fig. 4.

Evolutionary conservation of amino acid residues within selected segments of the HPS5 and HPS1 proteins. (A) Alignment of residues 139-152 from the human HPS5 protein isoform a (GenBank accession no. **NP_852608**) and corresponding segments within the orthologs from the chimpanzee (*Pan troglodytes*; GenBank accession no. **XP_508314**), horse (*Equus caballus*; GenBank accession no. **XP_001505011**), pig (*Sus scrofa*; GenBank accession no. **NP_001092073**), cattle (*Bos taurus*; GenBank accession no. **XP_869555**), dog (*Canis familiaris*; GenBank accession no. **XP_542523**), mouse (*Mus musculus*; GenBank accession no. **NP_001005247**), chicken (*Gallus gallus*; GenBank accession no. **XP_421011**), frog (*Xenopus tropicalis*; GenBank accession no. **NP_001090726**), zebrafish (*Danio rerio*;

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GenBank accession no. **AAI21197**), fugu fish (*Takifugu rubripes*; GenBank accession no. **DAA00973**), tetraodon (*Tetraodon nigroviridis*; GenBank accession no. **CAF95692**), fruit fly (*Drosophila melanogaster*; GenBank accession no. **NP_649810**) and red flour beetle (*Tribolium castaneum*; GenBank accession no. **XP_975447**). The conserved residue that is mutated in one allele of HPS patient 88 is denoted with an *arrow*. (B) Alignment of residues 536-557 from the human HPS1 protein isoform a (GenBank accession no. **NP_000186**) and corresponding protein segments from the chimpanzee (GenBank accession no. **XP_001165998**), orangutan (*Pongo pygmaeus*; GenBank accession no. **CAH92804**), macaque (*Macaca fascicularis*; GenBank accession no. **BAD51962**), pig (GenBank accession no. **NP_001092057**), horse (GenBank accession no. **XP_001500342**), dog (GenBank accession no. **XP_534985**), cattle (GenBank accession no. **ABH06318**), rat (*Rattus norvegicus*; GenBank accession no. **NP_414541**), mouse (GenBank accession no. **NP_062297**), opossum (*Monodelphis domestica*; GenBank accession no. **XP_001373344**), platypus (*Ornithorhynchus anatinus*; GenBank accession no. **XP_001508131**), chicken (GenBank accession no. **NP_001026751**), frog (GenBank accession no. **NP_001006903**), zebrafish (GenBank accession no. **NP_001032777**), tetraodon (GenBank accession no. **CAF89850**), red flour beetle (GenBank accession no. **XP_970712**), fruit fly (GenBank accession no. **NP_610997**) and honey bee (*Apis mellifera*; GenBank accession no. **XP_001121837**). The residues mutated in one allele of HPS patient 128 are denoted with *arrows*.
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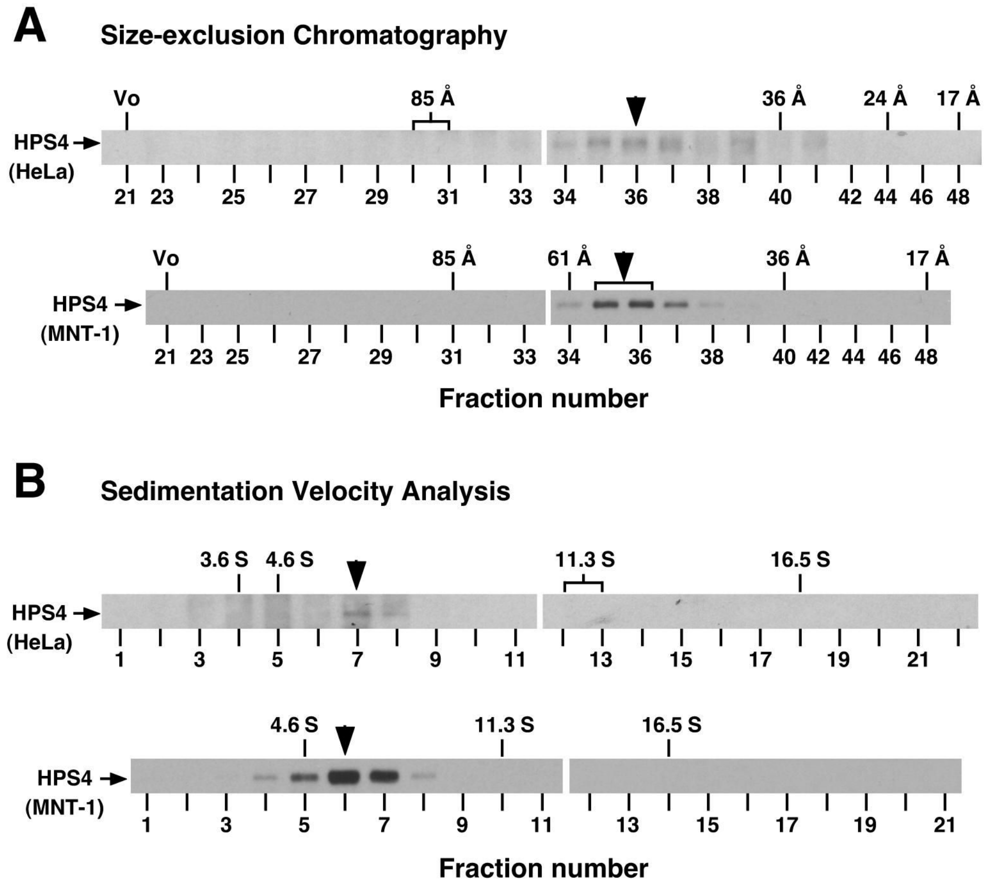

Fig. 5.

Estimation of the native molecular mass of human BLOC-3. (A) Size-exclusion chromatography. Freshly prepared cytosolic extracts from HeLa or pigmented melanoma MNT-1 cells were fractionated on a calibrated Superose 6 column (1 5 60 cm), and the resulting fractions analyzed by immunoblotting using the mAb against the HPS4 subunit of BLOC-3. The column's exclusion volume (V_0), and the elution positions of standard proteins (Stokes radii in Ångstroms) that were analyzed on the same column just before fractionation of each cytosolic sample, are indicated on the *top*. (B) Sedimentation velocity analysis. Freshly prepared cytosol from HeLa or MNT-1 cells was fractionated by ultracentrifugation for 16.5 and 13 h, respectively, on a 5-20% (w/v) sucrose gradient at 39,000 rpm in a SW41 rotor (~261,000 5 *g*), at 4°C. Fractions were collected from the bottom of the tube and analyzed by immunoblotting using mAb against HPS4. Fraction 1 corresponds to the top of the gradient. The positions of standard proteins (sedimentation coefficients in Svedbergs) that were analyzed in parallel with each cytosolic extract are indicated on the *top*.

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Table 1

Candidate genes for HPS and assembly of their products into protein complexes

HPS Disease	Gene Name	Number of Exons	Open Reading Frame[a] (bp)	Protein Complex
HPS-1	*HPS1*	20	2103	BLOC-3
HPS-2	*AP3B1*	27	3285	AP-3
HPS-3	*HPS3*	17	3015	BLOC-2
HPS-4	*HPS4*	14	2127	BLOC-3
HPS-5	*HPS5*	23	3390	BLOC-2
HPS-6	*HPS6*	1	2328	BLOC-2
HPS-7	*DTNBP1*	10	1056	BLOC-1
HPS-8	*BLOC1S3*	1	609	BLOC-1
?, [bc]	*PLDN*	5	519	BLOC-1
?, [bc]	*MUTED*	5	564	BLOC-1
?, [bc]	*CNO*	1	654	BLOC-1
?[c]	*SNAPAP*	4	411	BLOC-1
?[c]	*BLOC1S1*	4	378	BLOC-1
?[c]	*BLOC1S2*	5	429	BLOC-1
?, [bc]	*AP3D1*	30	3462	AP-3
?[c]	*AP3M1*	10	1257	AP-3
?[c]	*AP3S1*	6	582	AP-3
?[c]	*AP3S2*	6	582	AP-3

[a]Size of the longest predicted open reading frame, including termination codon.

[b]Not yet associated with HPS in humans, but mouse ortholog mutated in an inbred strain displaying HPS-like phenotypes.

[c]Protein assembled into a protein complex containing at least one HPS-associated gene product.

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Table 2

Sensitivity and variability in the detection of AP-3 and BLOC subunits in normal fibroblasts

Complex	Subunit	Apparent Mass (kDa)[a]	Sensitivity (μg total protein)	Variation Coefficient (%)[b]
AP-3	μ3A	~47	~1	41
BLOC-1	pallidin	~24	~3	22
BLOC-2	HPS6	~90	~1	18
BLOC-3	HPS4	~100	~10	52

[a] Apparent mobility on SDS-polyacrylamide gel electrophoresis

[b] Sample-to-sample variation in the signals obtained for whole-cell extracts (10 μg total protein) prepared from the skin fibroblasts of apparently healthy individuals.