BLOC-1, a Novel Complex Containing the Pallidin and Muted Proteins Involved in the Biogenesis of Melanosomes and Platelet-dense Granules*

Recent studies have led to the identification of a group of genes required for normal biogenesis of lysosome-related organelles such as melanosomes and platelet-dense granules. Two of these genes, which are defective in the pallid and muted mutant mouse strains, encode small, coiled-coil-forming proteins that display no homology to each other or to any known protein. We report that these two proteins, pallidin and muted, are components of a novel protein complex. We raised antibodies that allow for detection of pallidin from a wide variety of mammalian cells. Endogenous pallidin was distributed in both soluble and peripheral membrane protein fractions. Size-exclusion chromatography and sedimentation velocity analyses indicated that the bulk of cytosolic pallidin is a component of an asymmetric protein complex with a molecular mass of ~200 kDa. We named this complex BLOC-1 (for biogenesis of lysosome-related organelles complex 1). Steady-state pallidin protein levels were reduced in fibroblasts derived from muted and reduced pigmentation mice, suggesting that the genes defective in these two mutant strains could encode components of BLOC-1 that are required for pallidin stability. Co-immunoprecipitation and immuno-depletion experiments using an antibody to muted confirmed that this protein is a subunit of BLOC-1. Yeast two-hybrid analyses revealed that pallidin is capable of self-association through a region that contains its two coiled-coil forming domains. Unlike AP-3-deficient pearl fibroblasts, which display defects in intracellular zinc storage, zinc distribution was not noticeably affected in pallid or muted fibroblasts. Interestingly, immunofluorescence and in vitro binding experiments demonstrated that pallidin/BLOC-1 is able to associate with actin filaments. We propose that BLOC-1 mediates the biogenesis of lysosome-related organelles by a mechanism that may involve self-assembly and interaction with the actin cytoskeleton.

Lysosome-related organelles are a group of specialized, cell type-specific, membrane-bound compartments that share some characteristics with lysosomes, namely low luminal pH and the presence of lysosome-associated membrane proteins (LAMPS)\(^1\) in their limiting membrane (for recent reviews, see Refs. 1 and 2). Examples of lysosome-related organelles are melanosomes, platelet-dense granules, neutrophil azurophil granules, and lytic granules of cytotoxic T lymphocytes and natural killer cells. The functions of these organelles vary widely (1, 2), from synthesis and storage of melanin (melanosomes) and regulation of platelet aggregation (platelet-dense granules) to killing of invading bacteria (neutrophil azurophil granules) and virus-infected and tumor cells (lytic granules).

How are these organelles formed? One alternative is that a given lysosome-related organelle is actually the lysosome of a particular cell type, in which expression of cell type-specific proteins with lysosomal targeting information provides the organelle with functional properties different from, or in addition to, the degenerative role of conventional lysosomes. This appears to be the case for lytic granules and the major histocompatibility complex class II compartment (1). However, other lysosome-related organelles, such as melanosomes and platelet-dense granules, are known to co-exist with conventional lysosomes in the same cell (3, 4). Hence, sorting mechanisms must exist that ensure proper segregation of components of these organelles from those of lysosomes.

Some clues on the nature of the cellular machinery that mediates the biogenesis of melanosomes and platelet-dense granules (and, presumably, of other lysosome-related organelles) have arisen from the study of Hermansky-Pudlak syndrome (HPS). HPS defines a group of autosomal recessive disorders characterized by reduced pigmentation and prolonged bleeding due to defective melanosomes and platelet-dense granules, respectively (for a recent review, see Ref. 5). In humans, four forms of the disease, named HPS-1, -2, -3, and -4, have been associated with mutations in HPS1, AP3B1, HPS3, and HPS4, respectively (6–9). Of these genes, only AP3B1 encodes a protein of known function; this protein is the β3 subunit of the AP-3 sorting complex, which mediates signal-dependent trafficking of integral membrane proteins (e.g. the LAMPS) to organelles of the endosomal-lysosomal system (for a recent review, see Ref. 10). In mice, at least 15 non-allelic mutant strains display HPS-like combinations of hypopigmentation and platelet-dense granule deficiency (11). Four of these strains, termed pale ear, pearl, cocoa, and light ear, bear mutations in orthologues of the genes mutated in human HPS-1, -2, -3, and -4, respectively (9, 12–15). Affected genes have been identified for additional HPS-like mutants mouse strains, in-

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1 The abbreviations used are: LAMP(s), lysosome-associated membrane protein(s); 3AT, 3-amino-1,2,4-triazole; BLOC-1, biogenesis of lysosome-related organelles complex 1; BSA, bovine serum albumin; F-actin, filamentous actin; GST, glutathione S-transferase; HPS, Hermansky-Pudlak syndrome; PBS, phosphate-buffered saline; PA, pallidin.
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including mocha, gunmetal, pallid, and muted (16–19). Mocha mice are defective in another subunit of the AP-3 sorting complex, named δ (16). The gene defective in gunmetal mice encodes the α subunit of Rab geranylgeranylationtransferase, which prenylates small GTPases of the Rab family to facilitate their function in membrane trafficking (17). The genes affected in pallid and muted mice, named Pldn and me, respectively, encode novel proteins (18, 19). The Pldn gene product, pallidin, was shown to interact with syntaxin 13 (18), a member of the syntaxin family of integral membrane proteins involved in membrane fusion (for a recent review on the physiological significance of this interaction, see Ref. 20). No other biochemical or functional properties of pallidin have been described.

In this work, we have characterized the endogenous pallidin protein from mammalian tissues and cultured cells. We found that pallidin exists in soluble and membrane-associated forms and that the soluble form is a component of a large protein complex, which we named BLOC-1 (for biogenesis of lysosome-related organelles complex 1). We identified mutated as another subunit of BLOC-1, and we obtained evidence suggesting that pallidin is capable of self-association. We also observed that intracellular zinc distribution in fibroblasts is less sensitive to BLOC-1 deficiency than to AP-3 deficiency. Finally, we found that pallidin/BLOC-1 can interact with actin filaments in vitro and in transfected cells.

Experimental Procedures

DNA Constructs—The plasmids pGST-PA, pGST-PA N, and pGST-PA C were generated by PCR amplification of cDNA segments encoding human pallidin residues 1–172 (full length), 1–62, and 62–172, respectively, followed by in-frame cloning into EcoRI-SulI sites of the pGEX-5X-1 vector (Amersham Biosciences). The same DNA inserts were subcloned into a modified version of the pET-30a (+) vector (kindly provided by Dr. Markus Boehm, National Institutes of Health) to generate histidine-tagged versions of the pallidin fragments, and into the yeast two-hybrid vectors pGBT9 and pGAD424 (CLONTECH) to generate in-frame fusions with Gal4 binding and activation domains, respectively. The plasmid pGST-MU was obtained by PCR amplification of the complete open reading frame of human mutant (GenBank™ accession number AF426344) followed by cloning into the EcoRI-SulI sites of pGEX-5X-1. The corresponding pHiP-PA plasmid (pHiP-PA was derived by subcloning into pET-30a (+) (Newagen), pGBT9, and pGAD424, respectively. The mammalian expression plasmid pMYC-PA was generated by PCR engineering (to fuse the Myc epitope at the amino terminus of full-length human pallidin) followed by cloning into the pCR3.1 vector (Invitrogen). Plasmids coding for ARF1Q71L fused to the Gal4 DNA binding domain and for GGA3 residues 1–172 fused to the Gal4 activation domain have been described before (21).

Recombinant Protein Expression and Purification—Plasmids encoding GST or histidine-tagged fusion proteins were transformed into Escherichia coli BL21-CodonPlus-(DE3)-RP (Stratagene). GST fusion proteins were affinity-purified after expression by using glutathione-Sepharose 4 Fast Flow (Amersham Biosciences). Recombinant histidine-tagged proteins, rHis-PA, rHis-PA N, and rHis-MU, were purified on TALON Superflow resin (CLONTECH). All recombinant proteins were dialyzed against 0.1 mM Hepes, pH 8.0, except for rHis-PA and rHis-PA C, which were dialyzed against 0.1 mM Na2CO3, pH 11, or 1% (w/v) Triton X-100. Resuspended membranes were incubated for 1 h at room temperature and then centrifuged at 4 °C for 90 min at 120,000 × g. Final supernatants and pellets were diluted to an equal volume with SDS-PAGE sample buffer and then analyzed by immunoblotting.

Size-exclusion Chromatography and Sedimentation Velocity Analysis—Size-exclusion chromatography was performed at 4 °C on a calibrated Superose 6 column (1 × 60 cm) connected to a Fast Protein Liquid Chromatography system (Amersham Biosciences). The column was equilibrated with buffer D (0.3 M Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM MgCl2, and protease inhibitor mixture) instead of buffer A. For membrane extraction experiments, HeLa microsomal membranes were resuspended with the aid of a Dounce homogenizer in buffer C (10 mM Hepes, pH 7.4, 50 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM MgCl2 containing protease inhibitor mixture and no additive (control) or either 0.8 M Tris-HCl, pH 7.5, 0.2 mM Na2CO3, pH 11, or 1% (w/v) SDS was included in the sample). Fractions (0.5 ml) were collected and analyzed by immunoblotting. Calibration of the column was performed using a blue dextran (to determine exclusion volume) and the following protein standards from Sigma (Stokes radii in parentheses): bovine thyroglobulin (85 Å), horse spleen apoferritin (61 Å), BSA (36 Å), carbonic anhydrase (24 Å), and horse heart cytochrome c (17 Å).

Sedimentation velocity analysis was carried out by ultracentrifugation on a linear 5–20% (w/v) sucrose gradient (12 ml) prepared in buffer D. Bovine liver cytosol was diluted with an equal volume of water prior to loading (1 mg protein/ml sample). Fractions (0.5 ml) were collected from the bottom of the tube and analyzed by immunoblotting. The following protein standards (Sigma) were used (Svedberg coefficients in parentheses): horse spleen apoferritin (16.5 S), bovine catalase (11.3 S), BSA (4.6 S), and chicken ovomucoid (3.6 S).

Molecular mass and frictional ratio (f/f0) values were calculated from Stokes radii and sedimentation coefficients assuming a partial specific volume of 0.72–0.75 cm3/g, as described (24).

Immunoprecipitation and Immunoblotting—Immunoprecipitation-recapture experiments were carried out as described previously (25). Non-denaturing immunoprecipitations included one washing step using 0.1% (w/v) SDS, as described previously (25). Immunoblotting was performed as in a previous paper (22).

Yeast Two-hybrid Analysis—The Saccharomyces cerevisiae strain AH109 (CLONTECH) was transformed by following the lithium acetate method (26). Double transformants were selected in SD minimal medium (0.7% (w/v) yeast nitrogen base without amino acids (Sigma) and 2% (w/v) glucose, pH 5.5) supplemented with appropriate auxotrophic requirements except for leucine and tryptophan. At least two colonies of each double transformant were suspended in water at an absorbance of 0.4 to 0.6 mm at 550 nm. The resulting cell suspensions (5 μl) were spotted onto SD medium lacking leucine and tryptophan (control) or lacking also histidine and/or adenine, in the absence or presence of 5 mM 3AT. Cells were incubated at 30 °C for up to 3 days and monitored for growth by visual inspection.
**RESULTS**

**Characterization of New Antibodies to Pallidin**—We raised a rabbit polyclonal antisera against a recombinant GST fusion protein that composed the complete polypeptide chain predicted from the major transcript of the human PLDN gene (172 amino acid residues; GenBankTM accession number AF080470). The crude antisera was affinity-purified using as ligands a recombinant histidine-tagged form of pallidin, rHis-PA (to generate the anti-PA antibody), or recombinant proteins bearing pallidin residues 1–62 and 62–172 (to generate anti-PA\(_N\) and anti-PA\(_C\), antibodies, respectively). By immunoblotting, the three purified antibodies recognized rHis-PA as well as a polypeptide that was deemed to represent endogenous pallidin as it was present in wild-type mouse fibroblast extracts but absent from pallid fibroblast extracts (Fig. 1A). In addition, the antiserum allowed us to detect endogenous pallidin by immunoprecipitation. In the experiment shown in Fig. 1B, HeLa cells metabolically labeled with \([35S]\)methionine and \([35S]\)cysteine were lysed in the presence of non-ionic detergent and subjected to a first immunoprecipitation step (recapture) followed by SDS-PAGE and fluorography (right panel). Notice the presence of a single protein band in the sample immunoprecipitated in both steps with anti-pallidin. The approximate positions of molecular mass standards are indicated on the left.

**Actin-binding Assay**—Binding of proteins to F-actin was assayed using the Non-muscle Actin Binding Protein Biochem Kit from Cytoskeleton (Denver, CO). Briefly, purified actin was polymerized following the instructions provided. M1 cytosol prepared in buffer B was diluted with 2 volumes of buffer E (4.5 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM MgCl\(_2\), 1 mM ATP, and 0.18 mM CaCl\(_2\)) and centrifuged at 4°C for 90 min at 100,000 \(\times g\) to remove any potential aggregates. The resulting supernatant was incubated with F-actin in buffer E, or with buffer E alone, for 30 min at room temperature. Subsequently, each incubated sample (50 \(\mu\)l) was layered on top of 0.2 ml of cushion buffer (5 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl\(_2\), and 10% \(v/v\) glycerol) and centrifuged at room temperature for 90 min at 100,000 \(\times g\). The resulting pellets were analyzed by immunoblotting.

**Fluorescence Microscopy**—Cells were grown on glass coverslips to 50–80% confluence and fixed using 2.7% (v/v) formaldehyde in PBS. In some experiments, cells were transfected with expression plasmids using the FuGENE 6 reagent (Roche Molecular Biochemicals) about 24 h before fixation. All staining procedures were performed at room temperature. To detect Myc-PA, fixed cells were first incubated for 1 h with anti-Myc antibody diluted in buffer F (PBS containing 0.1% (w/v) saponin and 0.1% (w/v) BSA), washed in PBS, and then incubated for 30 min with Cy3-conjugated donkey anti-mouse IgG diluted in the same buffer. To stain actin filaments, fixed cells were incubated with Alexa Fluor 488 Phalloidin (Molecular Probes) diluted in buffer F. To detect intracellular zinc store sites, either fixed or unfixed cells were incubated for 1 h with 25 \(\mu\)M zinquin ethyl ester (Toronto Research Chemicals, Ontario, Canada) in Dulbecco’s modified Eagle’s medium containing 25 mM Hepes, pH 7.4, and 0.1% (w/v) BSA. Stained samples were washed extensively with PBS and mounted on glass slides using Fluromount-G (Southern Biotech, Birmingham, AL). Fluorescence microscopy was carried out on either Leica DMR or Leica TCS SP MP microscopes (Leica, Germany), as indicated in the legends to Figs. 7 and 8.

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Expression pattern and membrane association of pallidin. A, immunoblotting analysis of detergent extracts prepared from the indicated mouse tissues. Loading amounts were normalized by total protein concentration. Membranes were incubated with anti-PA to detect pallidin and with commercial antibodies to detect β-actin and α-tubulin. B, immunoblotting analysis of whole-cell extracts prepared from the human cell lines M1 (fibroblast), MNT-1 (pigmented melanoma), MEG-01 (megakaryoblastoid leukemia), and HeLa (epithelioid carcinoma). Loading amounts were normalized by cell number. C, HeLa cells and bovine kidney were homogenized in the absence of detergents and centrifuged at 15,000 × g to remove unbroken cells, nuclei, and mitochondria. The resulting supernatants were centrifuged at 120,000 × g to prepare cytosolic and microsomal membrane fractions. Samples were analyzed by immunoblotting using anti-PA antibody. D, microsomal membranes prepared from HeLa cells as in C were divided into aliquots and resuspended in buffer with or without the indicated additives. Subsequently, samples were centrifuged at 120,000 × g to separate supernatants (S) from pellets (P), which were analyzed by immunoblotting using anti-PA and an antibody to syntaxin 13 (Synt. 13). Notice that pallidin remained associated to the membrane pellet in the control sample but was completely solubilized with high salt (0.8 M Tris-HCl) or high pH (0.2 M Na2CO3, pH 11) and partially solubilized with detergent (1% Triton X-100). As expected, the integral membrane protein, syntaxin 13, was only solubilized by treatment with detergent.

In Fig. 1 strongly argue that the endogenous proteins detected by our antibodies represent bona fide mouse and human pallidin. Although alternatively spliced versions of pallidin mRNA have been identified and predicted to encode putative truncated forms of pallidin (18, 20), we have been unable to detect any of these putative forms by either immunoblotting or immunoprecipitation.

Expression Pattern and Association of Pallidin to Membranes—To study the expression pattern of pallidin at the protein level, we performed immunoblot analyses of extracts prepared from various mouse tissues (Fig. 2A) and human cell lines (Fig. 2B). In agreement with a previous mRNA expression analysis (18), the pallidin protein was detected in all tissues and cell lines examined. Interestingly, pallidin was not particularly abundant in pigmented cells (e.g. MNT-1 melanoma) or in megakaryoblastoid cell lines (e.g. MEG-01) despite the fact that the main manifestations of the mpd mutation in mice are hypopigmentation and platelet-dense granule deficiency (Fig. 2B).

Upon fractionation of crude cell extracts by differential centrifugation, the pallidin protein was consistently recovered from both cytosolic and microsomal membrane fractions (Fig. 2C). In addition, the membrane-associated form of pallidin could be quantitatively extracted using high salt concentrations or high pH (Fig. 2D), whereas a control integral membrane protein (syntaxin 13) remained associated to membranes under the same experimental conditions. Together, these results suggest that pallidin can exist as a soluble protein as well as a peripheral membrane protein.

Pallidin Is a Part of a Large Protein Complex—To estimate the size of the soluble form of pallidin under non-denaturing conditions, we performed size-exclusion chromatography and velocity density sedimentation analyses of bovine liver cytosol, and we used anti-PA for immunological detection of pallidin in the resulting fractions. Upon size-exclusion chromatography, cytosolic pallidin was resolved into two species (Fig. 3A). The most abundant species (fractions 30–31) eluted from the column in fractions corresponding to a Stokes radius of 89 ± 5 Å, whereas a minor population (fractions 40–41) eluted in fractions corresponding to 37 ± 6 Å (mean ± S.D. of 6 independent experiments). On the other hand, sedimentation velocity analyses performed by centrifugation on a linear sucrose concentration gradient did not allow for resolution of different pallidin species (Fig. 3B). Here the bulk of pallidin behaved as a protein with a sedimentation coefficient of 5.2 ± 0.3 S (mean ± S.D., n = 3). From these values, we calculated a molecular mass of 200 ± 30 kDa and a frictional ratio of ~2.3 for the major form of cytosolic pallidin. Consequently, these results suggest that the major soluble form of pallidin is a component of a large asymmetric protein complex. We named this novel complex BLOC-1.

Mutated Is a Subunit of BLOC-1—To identify additional components of BLOC-1, we examined by immunoblotting the steady-state levels of pallidin in skin fibroblasts derived from various mouse mutant strains that, like pallid, display hypopigmentation and platelet-dense granule deficiency. We reasoned that pallidin protein levels could be reduced in those mutant cells in which the affected gene encodes a subunit of BLOC-1 critical for complex assembly or stability. Such an effect has been documented for many other multisubunit complexes such as the heterotetrameric AP-3 adaptor complex, for example (7, 16, 27, 28). Pallidin levels were normal in fibroblasts from mocha (Fig. 4A) and pearl (data not shown) mice, which bear mutations in α1 and β3A subunits of the AP-3 complex, respectively (14, 16). On the other hand, the steady-state levels of the AP-3 α3 subunit were decreased in both mocha...
wild-type fibroblasts but not in muted fibroblasts (extracts prepared from wild-type and muted fibroblasts, using anti-rHis-MU of recombinant histidine-tagged muted (rHis-MU), and of whole-cell extracts prepared from wild-type and muted fibroblasts, using anti-muted antisera). Notice that the antisera recognized rHis-MU (apparent molecular mass ~33 kDa) and a protein that was present in wild-type fibroblasts but not in muted fibroblasts (arrow). Other protein bands revealed by the antisera likely represent nonspecific cross-reactivity.

(Fig. 4A) and data (not shown) mice, in agreement with previous studies (16, 27, 28). We also observed that pallidin protein levels were normal in cells from cocoa mice (data not shown), which bear a mutation in the recently identified HPS3 protein (15). In contrast, the steady-state levels of pallidin were shown), which bear a mutation in the recently identified HPS3 protein levels were normal in cells from cocoa mice (data not shown). We also observed that pallidin is not only undetectable in pallid fibroblasts but also significantly reduced in cell extracts from muted and reduced pigmentation (Red, pigm.) mice. Likewise, the muted protein is reduced or undetectable in the same three extracts. B, immunoblotting analysis of recombinant histidine-tagged muted (rHis-MU), and of whole-cell extracts prepared from wild-type and muted fibroblasts, using anti-muted antisera. Notice that the antisera recognized rHis-MU (apparent molecular mass ~33 kDa) and a protein that was present in wild-type fibroblasts but not in muted fibroblasts (arrow). Other protein bands revealed by the antisera likely represent nonspecific cross-reactivity.

To examine further whether muted could be a subunit of BLOC-1, we performed co-immunoprecipitation experiments by following the immunoprecipitation-recapture approach. Here a cleared lysate prepared from HeLa cells metabolically labeled with [35S]methionine and [35S]cysteine was immunoprecipitated using antibodies to pallidin, muted or, as controls, irrelevant antibodies, and an antibody to AP-3 α3. The immunoprecipitates were denatured, diluted in nondenaturing buffer, and subjected to recapture immunoprecipitation using a similar set of antibodies. The final immunoprecipitates were analyzed by SDS-PAGE and fluorography (Fig. 5A). As expected, each of the pallidin, muted, and AP-3 α3 proteins was recovered from samples in which the corresponding specific antibody had been used for both immunoprecipitation steps (Fig. 5A, lanes 2, 8, and 12, respectively). Importantly, muted was also recovered from the sample first immunoprecipitated with anti-pallidin (Fig. 5A, lane 4), and conversely, pallidin was recovered from the sample first immunoprecipitated with anti-muted (Fig. 5A, lane 10). Neither pallidin nor muted was found in samples first immunoprecipitated with irrelevant antibodies, anti-α3 or anti-HPS1p (Fig. 5A and data not shown). In addition, neither AP-3 α3 nor HPS1p was detected in samples that had been immunoprecipitated first with antibodies to pallidin or muted (Fig. 5A, lane 6, and data not shown). Interestingly, immunodepletion of pallidin from a cell extract resulted in the concomitant depletion of muted but not of AP-3 α3 (Fig. 5B). Taken together, these results demonstrate that pallidin and muted are associated into a stable complex.

**Palladin Is Capable of Self-association—** We next used the yeast two-hybrid system to determine whether association between pallidin and muted involved direct interaction between these two proteins. Human pallidin and muted were fused to either DNA binding or activation domains of the Gal4 transcription factor, and the resulting constructs were transformed into an appropriate yeast strain. Interactions were assayed by examining complementation of histidine and/or adenosine auxotrophy, in the absence or presence of 3AT. Under all conditions,
we failed to observe specific interaction between pallidin and muted. Surprisingly, co-expression of full-length pallidin fused to Gal4 DNA binding domain with the same protein fused to the Gal4 activation domain resulted in vigorous growth under all conditions tested, thus suggesting that pallidin can interact with itself, as the former was detected in the absence of histidine (Fig. 6A). Further yeast two-hybrid analyses using truncated forms of pallidin revealed that residues 62–172, which include two putative coiled-coil-forming domains, are both necessary and sufficient for self-association (Fig. 6B). Interaction of fragment 62–172 with itself or with full-length pallidin was, however, weaker than that of full-length pallidin with itself, as the former was detected in the absence of histidine (Fig. 6B) but not in the absence of adenine or in the presence of 3AT (data not shown).

Intracellular Zinc Distribution in Fibroblasts from Pallid and Muted Mice—A recent study (29) reported that storage of zinc within intracellular organelles is affected in fibroblasts from pearl mice, thus suggesting that AP-3 function is required, either directly or indirectly, for normal zinc homeostasis. Another AP-3 mutant mouse strain, mocha, displays inner ear defects that can be prevented by supplementing the diet of the mutant mice. Consistent with a cytosolic localization. However, high levels of expression of epitope-tagged pallidin, but not of irrelevant control constructs, resulted in significant co-localization with a network of actin filaments localized in the vicinity of the cell nucleus (Fig. 8, A and B, and data not shown). Co-localization of epitope-tagged pallidin with other types of actin filaments, such as long stress fibers, was less evident or negligible (Fig. 8, C and D). No gross changes in actin filament distribution were observed upon pallidin overexpression (data not shown).

Although the observed distribution of overexpressed pallidin might not faithfully reflect the localization of endogenous pallidin, it nonetheless implied that the protein might be capable of associating with F-actin. To test this idea, we incubated cytosol from human M1 cells with purified F-actin, isolated actin filaments from the mixture by ultracentrifugation on a glycerol cushion (to avoid contamination with soluble proteins), and analyzed the pellets by immunoblotting. As shown in Fig. 8E, about 10% of pallidin from the cytosolic extract was recovered from pellets containing F-actin, whereas it was not detected in control pellets obtained in the absence of F-actin. Under these conditions, other cytosolic proteins such as the α subunit of the AP-2 complex or HSP70 did not associate significantly with F-actin pellets (Fig. 8E). Similar results were obtained using bovine liver cytosol as the source of pallidin.
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In this paper we report that pallidin and muted, which are encoded by the genes defective in the HPS-like mouse strains pallid and muted, are components of a novel multisubunit protein complex named BLOC-1. Another protein complex implicated in the biogenesis of lysosomes and/or related organelles is AP-3, of which the β3 subunit is defective in HPS-2 patients and pearl mice, and the δ subunit is defective in mocha mice (7, 14, 16). In addition, the product of the gene mutated in HPS-1 patients and pale ear mice has been proposed to be a component of another protein complex (32), although it remains to be determined whether this complex represents a homo- or hetero-oligomer.

Evidence for the existence of BLOC-1 was first obtained through our characterization of endogenous pallidin from mammalian tissues and cell lines. By using our rabbit polyclonal antibodies to pallidin, we found that the protein exists in a soluble form and in a membrane-associated form that behaves as a peripheral membrane protein. Further analyses of the soluble form of endogenous pallidin indicated that it was associated into a complex having a molecular mass of about 200 kDa. A minor pallidin form of smaller size was detected by size-exclusion chromatography (Fig. 3A); it remains to be determined whether this minor form represents unassembled pallidin, a subcomplex of BLOC-1, or yet another pallidin-containing complex.

Immunoblotting analysis of fibroblasts from various HPS-like mutant mouse strains revealed that muted is required for normal steady-state levels of the pallidin protein (Fig. 4A). By using an antibody raised to muted, we were also able to show that pallidin is, in turn, required for normal muted steady-state protein levels. Moreover, stable physical association between these two proteins was demonstrated by co-immunoprecipitation as well as by co-immunodepletion (Fig. 5). We also observed that muted remained associated with pallidin upon fractionation of cytosol by centrifugation on a sucrose gradient. Low sensitivity of our antibody to muted precluded direct tests of the association of muted with one or both cytosolic pallidin species or with the membrane-bound form of pallidin. Nevertheless, our results are more consistent with the idea that BLOC-1 is a stable complex, thus implying that some of the biochemical properties reported herein for pallidin (e.g. association to membranes, cytoskeleton, and itself) may apply to the whole complex.

Despite the existence in pallidin and muted of domains with predicted propensity to form coiled-coil structures, we failed to detect direct interaction between these two proteins in the context of the yeast two-hybrid system. We also found no evidence of direct interaction between pallidin and muted upon co-expression of the two proteins in E. coli as combinations of GST and polystyrene fusion proteins.  

A possible explanation for these negative results is that BLOC-1 contains additional subunits required for assembly of pallidin and muted into the complex. Although the molecular mass previously estimated for the HPS1p-containing complex is similar to that of BLOC-1 (32), our co-immunoprecipitation experiments failed to detect association between pallidin and HPS1p, thus suggesting that the latter is not a subunit of BLOC-1. On the other hand, indirect evidence presented herein suggests the product of the rp gene as a candidate BLOC-1 subunit. Indeed, both pallidin and muted steady-state levels were drastically reduced in fibroblasts from reduced pigmentation mice, as we found in pallid and muted fibroblasts (Fig. 4A). However, a direct test of the possibility that rp encodes a subunit of BLOC-1 awaits identification of the rp gene. Additional candidate subunits of BLOC-1 are the proteins of apparent molecular masses ~87, ~76, ~46, and ~31 kDa that seemed to co-immunoprecipitate specifically with pallidin under non-denaturing conditions (Fig. 1B, arrows). Current efforts are aimed at identifying the remaining subunit(s) of BLOC-1.

An interesting property of pallidin is its tendency to self-

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**Fig. 8. Association of pallidin with F-actin.** A–D, HeLa cells grown on glass coverslips were transfected with a plasmid encoding a Myc epitope-tagged form of human pallidin, Myc-PA. About 24 h after transfection, cells were fixed with formaldehyde and stained with anti-Myc mouse monoclonal antibody followed by Cy3-conjugated donkey anti-mouse IgG (A and C) and, simultaneously, with Alexa-488-conjugated phallolidin (B and D). Serial images of Cy3 and Alexa 488 fluorescence were acquired on a Leica TCS SP MP confocal microscope using a 2-section thickness of about 0.4 μm. A and B correspond to an optical section just above the cell nucleus, whereas C and D correspond to another section of the same cell that includes the basal plasma membrane attached to the glass surface. Notice the extensive co-localization of Myc-PA and actin filaments in A and B (arrows), which is less evident in the lower section containing long actin stress fibers (C and D). Control experiments (not shown) corroborated the lack of optical cross-over between Cy3 and Alexa 488 fluorescence under these conditions, and confirmed that the perinuclear actin filament network shown in B was not an artifact of pallidin overexpression as it was also present in untransfected cells. E, binding of F-actin in vitro. Cytosol from M1 cells was incubated with (+) or without (−) polymerized non-muscle actin (F-actin) and subsequently ultracentrifuged on top of a glycerol cushion to sediment actin filaments and associated proteins. The resulting pellets, as well as an aliquot of the cytosol (1/3 total control), were analyzed by immunoblotting using antibodies to pallidin (anti-PA), the α subunit of AP-2, or HSP70. Notice the presence of pallidin in pellets containing F-actin.

(data not shown). Taken together, these observations suggest that pallidin is able to interact, directly or indirectly, with actin filaments.

**DISCUSSION**

In this paper we report that pallidin and muted, which are encoded by the genes defective in the HPS-like mouse strains pallid and muted, are components of a novel multisubunit protein complex named BLOC-1. Another protein complex implicated in the biogenesis of lysosomes and/or related organelles is AP-3, of which the β3 subunit is defective in HPS-2 patients and pearl mice, and the δ subunit is defective in mocha mice (7, 14, 16). In addition, the product of the gene mutated in HPS-1 patients and pale ear mice has been proposed to be a component of another protein complex (32), although it remains to be determined whether this complex represents a homo- or hetero-oligomer.

Evidence for the existence of BLOC-1 was first obtained through our characterization of endogenous pallidin from mammalian tissues and cell lines. By using our rabbit polyclonal antibodies to pallidin, we found that the protein exists in a soluble form and in a membrane-associated form that behaves as a peripheral membrane protein. Further analyses of the soluble form of endogenous pallidin indicated that it was associated into a complex having a molecular mass of about 200 kDa. A minor pallidin form of smaller size was detected by size-exclusion chromatography (Fig. 3A); it remains to be determined whether this minor form represents unassembled pallidin, a subcomplex of BLOC-1, or yet another pallidin-containing complex.

Immunoblotting analysis of fibroblasts from various HPS-like mutant mouse strains revealed that muted is required for normal steady-state levels of the pallidin protein (Fig. 4A). By using an antibody raised to muted, we were also able to show that pallidin is, in turn, required for normal muted steady-state protein levels. Moreover, stable physical association between these two proteins was demonstrated by co-immunoprecipitation as well as by co-immunodepletion (Fig. 5). We also observed that muted remained associated with pallidin upon fractionation of cytosol by centrifugation on a sucrose gradient. Low sensitivity of our antibody to muted precluded direct tests of the association of muted with one or both cytosolic pallidin species or with the membrane-bound form of pallidin. Nevertheless, our results are more consistent with the idea that BLOC-1 is a stable complex, thus implying that some of the biochemical properties reported herein for pallidin (e.g. association to membranes, cytoskeleton, and itself) may apply to the whole complex.

Despite the existence in pallidin and muted of domains with predicted propensity to form coiled-coil structures, we failed to detect direct interaction between these two proteins in the context of the yeast two-hybrid system. We also found no evidence of direct interaction between pallidin and muted upon co-expression of the two proteins in E. coli as combinations of GST and polystyrene fusion proteins.

A possible explanation for these negative results is that BLOC-1 contains additional subunits required for assembly of pallidin and muted into the complex. Although the molecular mass previously estimated for the HPS1p-containing complex is similar to that of BLOC-1 (32), our co-immunoprecipitation experiments failed to detect association between pallidin and HPS1p, thus suggesting that the latter is not a subunit of BLOC-1. On the other hand, indirect evidence presented herein suggests the product of the rp gene as a candidate BLOC-1 subunit. Indeed, both pallidin and muted steady-state levels were drastically reduced in fibroblasts from reduced pigmentation mice, as we found in pallid and muted fibroblasts (Fig. 4A). However, a direct test of the possibility that rp encodes a subunit of BLOC-1 awaits identification of the rp gene. Additional candidate subunits of BLOC-1 are the proteins of apparent molecular masses ~87, ~76, ~46, and ~31 kDa that seemed to co-immunoprecipitate specifically with pallidin under non-denaturing conditions (Fig. 1B, arrows). Current efforts are aimed at identifying the remaining subunit(s) of BLOC-1.

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associate, which was inferred from yeast two-hybrid experiments using full-length and truncated versions of pallidin. Self-association was observed for the full-length protein as well as residues 62–172, which contain two predicted coiled-coil forming domains. This phenomenon may explain the high tendency of recombinant proteins having this fragment to form aggregates at physiological pH and ionic strength. It will now be important to determine whether BLOC-1, not just pallidin, is capable of self-assembly and whether this property is indeed relevant to BLOC-1 function.

What is the function of BLOC-1? Both pallid and muted mice display severely reduced coat and eye pigmentation, prolonged bleeding due to storage pool deficiency, impaired secretion of lysosomal enzymes from kidney and platelets, and, with low penetrance, inner ear defects due to abnormal otolith formation (reviewed in Refs. 11 and 20). Pallid mice have also been reported to slowly develop emphysematous lung lesions (reviewed in Refs. 11 and 20). Although the implications of otolith defects and lung lesion development for BLOC-1 function remain obscure, it seems reasonable to assume that the complex is involved in the biogenesis of melanosomes and platelet-dense granules as well as in the biogenesis or dynamics of other organelles of the lysosomal system.

As mentioned above, the AP-3 sorting complex has also been implicated in the biogenesis of melanosomes, platelet-dense granules, and other organelles of the lysosomal system. Both pearl and mocha mutations in mice, as well as HPS-2 disease in humans, cause hypopigmentation and storage pool deficiency (7, 14, 16). As in pallid and muted mice, kidney and platelet lysosomal enzyme secretion is reduced in both mocha and pearl mice (reviewed in Ref. 11). Mocha mice also display inner ear defects similar to those of pallid and muted mice (11); these abnormalities are not observed in pearl mice or HPS-2 patients, presumably because the AP-3 β3A mutation can be complemented in brain by expression of a tissue-specific isoform named β3B (reviewed in Ref. 10). These considerations led us to consider the possibility that BLOC-1 could function in AP-3-dependent protein trafficking events. However, evidence reported here, together with that of previous studies (23, 33), suggests that BLOC-1 function is independent of AP-3. First, we failed to detect physical interaction between BLOC-1 and AP-3 by co-immunoprecipitation (Fig. 5) or affinity GST pull-down assays. Second, the phenotype of mutated/pearl homozygous double mutants is significantly more severe than those of mutated or pearl single mutants (33). Third, pallid and muted fibroblasts do not display the enhanced plasma membrane trafficking of LAMPs observed for pearl and mocha fibroblasts (23). Finally, the abnormal zinc distribution displayed by pearl fibroblasts (29) was not observed for pallid or muted fibroblasts (Fig. 7).

One potential clue for the cellular function of BLOC-1 is the reported interaction of pallidin with syntaxin 13, which would imply a role in membrane fusion (18). Another potential clue stems from our observation that pallidin is capable of associating with actin filaments. Epitope-tagged forms of pallidin localized to a subset of actin filaments upon overexpression in HeLa cells. Although the distribution of overexpressed pallidin may not reflect that of endogenous BLOC-1, it nonetheless suggests that pallidin is able to associate with F-actin. In strong support of this idea, endogenous pallidin (likely as part of BLOC-1) from M1 and bovine liver cytosols specifically associated to purified F-actin in an in vitro binding assay (Fig. 8E and data not shown). It remains to be determined whether association of pallidin/BLOC-1 to F-actin is direct or mediated by other actin-binding proteins.

Accumulating evidence indicates important roles for the actin cytoskeleton in organelle dynamics and membrane trafficking (reviewed in Refs. 34–37). The cytoplasmic aspect of the plasma membrane, and of some intracellular organelles such as the Golgi complex, has an associated skeleton containing actin and actin-binding proteins (e.g., spectrin and ankyrin). Conceivably, these actin-based membrane skeletons must be rearranged to allow for exit and arrival of membrane-bound transport intermediates (e.g., vesicles) that ferry lipids and proteins from and to each compartment (36–38). Accordingly, regulatory proteins such as Cad24 have been shown to induce actin rearrangements, at both the Golgi complex and plasma membrane, concomitantly to vesicle-mediated trafficking (37, 39). In addition, actin-rich structures known as “comet tails” have been found to associate with moving phagosomes and endocytic vesicles (40, 41), and an actin-based motor, myosin VI, has been directly implicated (42) in movement of endocytic vesicles (note, however, that other types of transport intermediates have been shown to move independently of the actin cytoskeleton; see for example Ref. 43). Finally, actin filaments can cooperate with microtubules for the movement and localization of whole organelles, as has been documented for melanosomes (44) and lysosomes (45). Consequently, a plethora of possible functions for BLOC-1 in actin-dependent events would be consistent with a role in the biogenesis of lysosome-related organelles. A challenge for future studies will be to test these possibilities to establish the exact function of BLOC-1.

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