BLOC-1 Is Required for Cargo-specific Sorting from Vacuolar Early Endosomes toward Lysosome-related Organelles[□]

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Hermansky-Pudlak syndrome (HPS) is a genetic disorder characterized by defects in the formation and function of lysosome-related organelles such as melanosomes. HPS in humans or mice is caused by mutations in any of 15 genes, five of which encode subunits of biogenesis of lysosome-related organelles complex (BLOC)-1, a protein complex with no known function. Here, we show that BLOC-1 functions in selective cargo exit from early endosomes toward melanosomes. BLOC-1-deficient melanocytes accumulate the melanosomal protein tyrosinase-related protein-1 (Tyrp1), but not other melanosomal proteins, in endosomal vacuoles and the cell surface due to failed biosynthetic transit from early endosomes to melanosomes and consequent increased endocytic flux. The defects are corrected by restoration of the missing BLOC-1 subunit. Melanocytes from HPS model mice lacking a different protein complex, BLOC-2, accumulate Tyrp1 in distinct downstream endosomal intermediates, suggesting that BLOC-1 and BLOC-2 act sequentially in the same pathway. By contrast, intracellular Tyrp1 is correctly targeted to melanosomes in melanocytes lacking another HPS-associated protein complex, adaptor protein (AP)-3. The results indicate that melanosome maturation requires at least two cargo transport pathways directly from early endosomes to melanosomes, one pathway mediated by AP-3 and one pathway mediated by BLOC-1 and BLOC-2, that are deficient in several forms of HPS.

INTRODUCTION

Hermansky-Pudlak syndrome (HPS) is a genetic disorder characterized by hypopigmentation, prolonged bleeding, and sometimes ceroid accumulation, lung fibrosis, and/or immune defects leading to premature death (Li *et al.*, 2004; Wei, 2006). HPS or a similar disorder in mice results from mutations in any of at least 15 genes (Wei, 2006). All of these genes are ubiquitously expressed, but their mutation in HPS

affects mainly the generation and function of selected tissue-specific lysosome-related organelles (LROs; Bonifacino, 2004; Di Pietro and Dell'Angelica, 2005). Those LROs that are most severely affected in all forms of HPS—pigment cell melanosomes, platelet dense granules, and lung lamellar bodies—are unique in that they coexist with bona fide lysosomes in their respective cell types (Dell'Angelica *et al.*, 2000; Marks and Seabra, 2001). The 15 known HPS-associated genes have been identified, and although the products of most are thought to participate in trafficking events that are uniquely required to form this class of LRO, the function of only a few is understood in detail.

The genes disrupted in human HPS-7 (Li *et al.*, 2003) and HPS-8 (Morgan *et al.*, 2006) and in the mouse HPS models *pallid*, *muted*, *reduced pigmentation* (*rp*), *cappuccino*, and *sandy* encode five of the eight known subunits of a stable protein complex known as biogenesis of lysosome-related organelles complex (BLOC)-1 (Falcon-Perez *et al.*, 2002; Moriyama and Bonifacino, 2002; Ciciotte *et al.*, 2003; Li *et al.*, 2003; Gwynn *et al.*, 2004; Starcevic and Dell'Angelica, 2004). To date, no specific subcellular function has been assigned to BLOC-1. A role for BLOC-1 in synaptic function is suggested by a putative, but disputed (Vites *et al.*, 2004), interaction of its snapin subunit with the target membrane-associated soluble *N*-ethylmaleimide-sensitive factor attachment protein

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Abbreviations used: BLOC, biogenesis of lysosome-related organelles complex; HPS, Hermansky-Pudlak syndrome; IEM, immunoelectron microscopy; IFM, immunofluorescence microscopy; LRO, lysosome-related organelle; MuHA, HA11-epitope tagged muted; PaHA, HA11-epitope tagged pallidin; *rp*, reduced pigmentation; syn13, syntaxin 13; Tf, transferrin; TfR, transferrin receptor; Tyrp1, tyrosinase-related protein-1.

receptor (tSNARE) synaptosome-associated protein of 25 kDa (SNAP-25) and its nonneuronal paralogue SNAP-23 (Ilardi *et al.*, 1999; Buxton *et al.*, 2003), by the linkage of dysbindin subunit gene polymorphisms with schizophrenia (Straub *et al.*, 2002; Numakawa *et al.*, 2004), and by the copurification of BLOC-1 with synaptic vesicles from PC12 cells (Salazar *et al.*, 2005). BLOC-1 in nonneuronal cells has been suggested to function on endosomes based on the interaction of its pallidin subunit with the endosomal tSNARE, syntaxin 13 (syn13; Huang *et al.*, 1999; Moriyama and Bonifacino, 2002), and on altered intracellular distribution or cell surface accumulation of endosomal proteins in BLOC-1-deficient fibroblasts (Di Pietro *et al.*, 2006; Salazar *et al.*, 2006). In melanocytes, a cell type affected by HPS, BLOC-1 localizes at steady state to a subdomain of early endosomes and influences the cell surface flux of a melanosomal protein, tyrosinase-related protein-1 (Typr1; Di Pietro *et al.*, 2006). Despite these observations, neither the pathway by which BLOC-1-dependent cargoes travel nor the specific transport step regulated by BLOC-1 is known. Furthermore, although BLOC-1 interacts physically with two other protein complexes—BLOC-2 and adaptor protein (AP)-3—that are defective in different forms of HPS (Di Pietro *et al.*, 2006), and AP-3 regulates cargo transport in melanocytes (Huizing *et al.*, 2001; Theos *et al.*, 2005), a functional link between these complexes has not been well established. Here, we exploit primary and immortalized melanocytes from HPS model mice to identify a vesicular transport step regulated by BLOC-1 that is required for trafficking of selected cargo from early endosomes to maturing melanosomes. We also provide evidence that BLOC-2, but not AP-3, functions in the same pathway.

MATERIALS AND METHODS

Reagents and Fluorescent Probes

Chemicals were from Sigma-Aldrich (St. Louis, MO) except where noted. Tissue culture reagents were from Invitrogen (Carlsbad, CA). FuGENE-6, hygromycin B, and protease inhibitor cocktail were from Roche Diagnostics (Indianapolis, IN). Mouse transferrin-fluorescein isothiocyanate (Tf-FITC) was from United States Biological (Swampscott, MA). Protein A conjugated to 10- or 15-nm gold particles was from Cell Microscopy Center (AZU, Utrecht, The Netherlands).

Antibodies

Monoclonal antibodies, their targets, and sources were as follows: TA99 to Typr1 and 7G7.B6 to Tac were from American Type Culture Collection (Manassas, VA); YOL1/2 to tubulin was from Santa Cruz Biotechnology (Santa Cruz, CA); 1D4B to mouse lysosomal-associated membrane protein (LAMP)-1 and H4A3 to human LAMP-1 were from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); HMB-45 and HMB-50 to human Pmel17 were from Lab Vision (Fremont, CA); anti-mouse Tf receptor (CD71) was from BD Transduction Laboratories (San Diego, CA); H68.4 anti-human Tf receptor was from Zymed Laboratories (South San Francisco, CA); anti-HA.11 was from Covance Research Products (Berkeley, CA); and anti- δ adaptin (Peden *et al.*, 2004) was a gift from A. Peden (Cambridge University, Cambridge, United Kingdom). Polyclonal rabbit antisera to pallidin (Moriyama and Bonifacino, 2002) (a gift from J. Bonifacino, National Institutes of Health, Bethesda, MD), dysbindin and BLOS3 subunits of BLOC-1 (Starcevic and Dell'Angelica, 2004), syn13 (Prekeris *et al.*, 1998), tyrosinase (Theos *et al.*, 2005), and mouse Pmel17 (Theos *et al.*, 2006) have been described previously. Goat anti-EEA1, rabbit anti-Myc and H-90 rabbit anti-Typr1 (used for immunoblotting) were from Santa Cruz Biotechnology. FITC-, rhodamine red-X-, and 7-amino-4-methylcoumarin-3-acetic acid-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA); rabbit anti-Alexa-488-, anti-FITC-, and Alexa-488-conjugated secondary antibodies were from Invitrogen. Conjugation of Alexa-488 or R-phycoerythrin (R-PE) to monoclonal antibodies was performed using protein labeling kits from Invitrogen or Prozyme (San Leandro, CA), respectively.

DNA Constructs

Retroviral constructs encoding human HA11-epitope tagged pallidin (PaHA) and HA11-epitope tagged muted (MuHA) were generated by subcloning

XhoI-NotI inserts from pXS vectors (Moriyama and Bonifacino, 2002), via pCI-neo intermediates, into pBMN-IRES-Hygro (a gift from R. Scheller, Genentech, San Francisco, CA). Untagged or Myc-epitope-tagged constructs were generated the same way, and untagged human BLOS3 was amplified from I.M.A.G.E. clone 3627463 (GenBank AY531266) and subcloned into the BamHI and XhoI sites of pBMN-IRES-Hygro. Mutagenesis of C-terminal amino acids L514A, L515A, and Y526A of Typr1 (Typr1 LLY), subcloned as a EcoRI fragment from pCDNA3-TRP-1 (a gift from Dr. W. Storkus, University of Pittsburgh, Pittsburgh, PA) in pCDM8.1 (Marks *et al.*, 1996), was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All plasmid inserts were verified by DNA sequencing.

Cell Culture and Transgene Expression

Primary melanocytes were isolated from neonatal C57BL/6J (wild type) and *pallid* (B6.Cg-*Pldn*^{Pa}/J, formerly C57BL/6J-*pa*) mice as described previously (Sviderskaya *et al.*, 1997) and used within the first three passages. Immortal melanocyte cell lines melan-mu1, -2, and -3 and melan-pa1, -2, and -3 were grown from skins of neonatal *muted* (*Muted*^{mu/mu}) *Ink4a*-Arf-/-B6.CHMU/Le and *pallid* *Ink4a*-Arf-/- mice, respectively, as described previously (Sviderskaya *et al.*, 2002). Melan-mu3 (referred to as melan-mu), melan-rp2 (Gwynn *et al.*, 2004) (referred to as melan-rp; *Bloc1s3*^{rp/rp}), melan-pa1 (referred to as melan-pa), melan-coa2 (Suzuki *et al.*, 2001) (referred to as melan-coa; *Hps3*^{coa/coa}), and melan-a (Bennett *et al.*, 1987) were maintained as described previously (Sviderskaya *et al.*, 2002). Retrovirus production from transiently transfected 293T cells and transduction of primary melanocytes and cell lines were carried out as described previously (Swift *et al.*, 1999). Stable transductants were selected in medium containing 200–400 μ g/ml hygromycin B. The 1011-mel cells were maintained as described previously (Berson *et al.*, 2001), transiently transfected using FuGENE-6 with 3 μ g of DNA in a six-well dish, and analyzed 48 h after transfection.

Immunofluorescence Microscopy and Immunoblotting

Cells were fixed with 2% formaldehyde, labeled with primary and fluorochrome-conjugated secondary antibodies as described previously (Berson *et al.*, 2001), and analyzed on a DM IRBE microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Orca digital camera (Hamamatsu, Bridgewater, NJ). Images were captured and manipulated using OpenLab software (Improvision, Lexington, MA) with the volume deconvolution package; regions were magnified for insets by using Adobe Photoshop (Adobe Systems, Mountain View, CA). To quantify marker overlap, deconvolved paired images were rendered binary by density slicing and the total area of overlap between them was calculated for objects containing more than 5 pixels, excluding the densely labeled perinuclear area. Eight to 14 cell profiles comprising >10,000 objects were quantified for each pairwise comparison. For cell surface labeling, cells on coverslips were incubated with medium containing Alexa-488-conjugated TA99 for 30–45 min at 4°C, washed with ice-cold PBS, and then fixed as described above. For immunoblots, cells were lysed in buffer (100 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1% SDS, and protease inhibitor cocktail), sonicated for 1–2 min in a water bath sonicator, clarified by centrifugation, fractionated, and immunoblotted as described previously (Berson *et al.*, 2001). Immunoblots were developed with enhanced chemo fluorescence (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and analyzed using a STORM PhosphorImager and ImageQuant software (GE Healthcare).

Electron Microscopy

Tf-FITC was internalized for 15 min as described previously (Theos *et al.*, 2005). Cells were fixed with 2% (wt/vol) paraformaldehyde with or without 0.2% (wt/vol) glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4, and processed for ultracycromicrotomy as described previously (Raposo *et al.*, 1997). Ultrathin cryosections were prepared with an ultracycromicrotome Ultracut FCS (Leica, Vienna, Austria), and single or double immunogold labeled using protein A conjugated to 10- or 15-nm gold (PAG10, PAG15). Sections were analyzed under a Philips CM120 electron microscope (FEI, Eindhoven, The Netherlands), and digital acquisitions were made with a numeric camera Keen View (Soft Imaging System, Munster, Germany). Quantification of Typr1 and tyrosinase distribution on the different intracellular compartments was performed as described previously (Theos *et al.*, 2005).

Flow Cytometry

Cell Surface Levels. Cells were harvested in Trypsin-EDTA, washed and suspended in growth medium (with 10% fetal bovine serum [FBS], 1% L-glutamine, and 25 mM HEPES, pH 7.4) containing saturating concentrations of unconjugated or Alexa-488-conjugated primary antibody on ice for 30–45 min. Cells were washed twice with ice-cold growth medium, incubated for 30 min on ice with Alexa-488-conjugated secondary antibody, and washed again where necessary, and then resuspended in ice-cold fluorescence-activated cell sorting (FACS) buffer (5% FBS, 1 mM EDTA, and 0.02% sodium azide in PBS) for analysis on a FACScan Plus by using CellQuest Pro software (BD Biosciences). Mean fluorescence intensity (MFI) was calculated by subtracting a

Figure 1. Melanin synthesis is inhibited in BLOC-1 mutant melanocytes but restored by reexpression of the missing BLOC-1 subunit. (a–e) Bright field microscopy of primary melanocytes (a–c) from wild-type C57BL/6J (a) or *pallid* mice (b and c), or immortalized melan-mu cells (d and e). (c and e) Cells were infected with recombinant retroviruses expressing PaHA (c) or MuHA (e). Images were taken at identical lighting and camera settings. Bar, 10 μ m. (f and g) Whole cell lysates of melan-mu or melan-mu:MuHA were fractionated by SDS-PAGE and immunoblotted with antibodies to the HA epitope (f) or the BLOC-1 subunits pallidin, BLOS3, or dysbindin (g),

and reprobbed with anti-tubulin as a loading control. Left, migration of molecular weight markers (in kilodaltons). Arrows indicate relevant bands. Note the reduced levels of dysbindin, BLOS3, and pallidin in melan-mu relative to melan-mu:MuHA.

background value obtained with 7G7.B6 anti-Tac antibody from the value of experimental samples. Melan-mu and melan-rp values were normalized to those of melan-mu:MuHA and melan-rp:BLOS3, respectively, within each experiment.

Endocytosis. Cells in suspension were incubated with growth medium containing unconjugated primary antibody on ice for 30 min, washed twice with ice-cold growth medium, and then incubated at 37°C to allow internalization. At each time point, samples were transferred to ice and Alexa-488-conjugated secondary antibodies were added to detect remaining cell surface molecules. Samples were washed twice with ice-cold medium, suspended in FACS buffer, and analyzed as described above. The % starting MFI for each experiment was calculated by dividing the MFI value of each time point with that of the zero time point. Endocytic rates were calculated from mean % starting MFI values within the first 2 min of internalization (for which the data of all samples could be reasonably fitted to a single exponential decay) by a log regression analysis by using LOGEST in Microsoft Excel (Microsoft, Redmond, WA).

Recycling. Tyrp1 recycling was measured essentially as described previously (Peden *et al.*, 2004). Briefly, cells were incubated with growth medium containing Alexa-488-conjugated TA99 antibody (or 7G7.B6 as a control) on ice for 30 min, washed twice with ice-cold growth medium, and incubated at 37°C for 5 min to internalize antibody-bound Tyrp1. Cells were then incubated on ice with rabbit anti-Alexa-488 antibody to quench remaining cell surface fluorescence, washed twice with ice-cold growth medium, and divided into two pools. Cells were incubated for indicated times at 37°C in medium containing either rabbit anti-Alexa-488 or rabbit anti-Myc as a control, washed twice with ice-cold medium, suspended in FACS buffer, and analyzed as described above. The % starting MFI values at each time point for samples with anti-Alexa-488 were normalized to those with anti-Myc (normalized % MFI). Recycling rates were calculated from mean % starting MFI values within the first 10 min of recycling by log regression analysis. Identical results were obtained using a Fab fragment of TA99 generated with the ImmunoPure Fab preparation kit (Pierce Chemical, Rockford, IL).

For Tf receptor recycling, cells were washed, resuspended in serum-free medium containing 0.1% BSA, and starved for 30 min at 37°C. Cells were then incubated in the same medium containing 50–60 μ g/ml Tf-FITC for 30 min at 37°C to allow Tf-FITC uptake, washed, and suspended in growth medium containing 10% FBS. Then, cells were incubated for the indicated times at 37°C to release the recycled Tf-FITC to the medium. Samples were washed twice with ice-cold medium, suspended in FACS buffer, and analyzed as described above.

Cell Surface Delivery. Cells were incubated with growth medium containing Alexa-488-conjugated TA99 on ice for 30 min, then additionally at 37°C for the indicated times. Cells were then washed twice with ice-cold medium, suspended in FACS buffer and analyzed as described above. In some experiments, cells were preincubated with unconjugated TA99 for 15 min on ice and 37°C for 20 min before washing twice and incubation with Alexa-488-conjugated antibody to saturate the cohort of recycling Tyrp1. For brefeldin A (BFA)-treated samples, 10 μ g/ml BFA was added to medium during a 60-min preincubation at 37°C and to all incubation and wash media.

Endocytosis Competition. The experiments were essentially as described previously (Marks *et al.*, 1996). Briefly, 1011-mel cells were transfected with pCDM8.1, pCDM8.1-Tyrp1(WT), or pCDM8.1-Tyrp1(LLY). Cells were harvested 48 h after transfection and divided into two pools. Samples were incubated on ice for 30–45 min in growth medium containing R-PE-conjugated TA99 with either H4A3 anti-LAMP-1- or Alexa-488-conjugated HMB-50. Cells labeled with H4A3 were washed twice with growth medium and incubated in growth medium containing Alexa-488-conjugated isotype-specific anti-IgG1 antibody on ice for 30–45 min. All samples were washed twice with growth medium, resuspended in FACS buffer, and analyzed as described above.

RESULTS

Pigmentation Defects in BLOC-1-deficient Melanocytes Are Corrected by Rescue with the Missing BLOC-1 Subunit

Primary (Figure 1a) or immortalized (Bennett *et al.*, 1987) melanocytes from wild-type C57BL/6J mice harbor highly pigmented melanosomes, visible by bright field microscopy. By contrast, nearly no pigment was observed in primary melanocytes from *pallid* mice, which lack the BLOC-1 subunit pallidin (Figure 1b), or in immortal melanocytes generated from *muted* (melan-mu; Figure 1d), *pallid* (melan-pa; see Figure 9c), or *rp* (melan-rp; our unpublished data) mice, which lack the BLOC-1 subunits muted, pallidin, and BLOS3, respectively. Modest pigmentation of primary *pallid* melanocytes developed only with increasing passage, likely due to senescence (Sviderskaya *et al.*, 2002). On infection of early passage *pallid* melanocytes with recombinant retroviruses expressing hemagglutinin (HA)-epitope-tagged pallidin (PaHA), but not HA-tagged muted, ~10–15% of the cells developed pigment within 3–5 d (Figure 1c). Similarly, pigmentation developed in melan-mu cells stably reexpressing wild-type muted with an HA- (MuHA; Figure 1, e and f) or myc-epitope tag or no tag (our unpublished data), but not PaHA, and in melan-rp and melan-pa cells by stably reexpressing BLOS3 (our unpublished data) and PaHA, respectively (Supplemental Figure S4). MuHA expression in melan-mu cells stabilized the additional BLOC-1 subunits pallidin, dysbindin, and BLOS3 (Figure 1g), indicating reconstitution of the BLOC-1 holocomplex. These results establish that hypopigmentation of *muted*, *pallid*, and *rp* melanocytes is a direct consequence of the deficiency in BLOC-1 and can be rapidly restored upon restoration of the complex.

Mislocalization of Tyrp1 in BLOC-1-deficient Melanocytes to Early Endosomes

To test whether BLOC-1 regulates protein transport to melanosomes, we screened BLOC-1-deficient melanocytes by immunofluorescence microscopy (IFM) for steady-state distribution of several melanosomal cargoes relative to endosomal proteins. Among the cargoes analyzed (Supplemental Figures S1 and S2), Tyrp1 was the most dramatically mislocalized in BLOC-1-deficient cells. Tyrp1 in control cells localized primarily to mature stage III and IV melanosomes, apparent by IFM as “donuts” filled with dark melanin (Figures 2a and 3a). By contrast, Tyrp1 was exclusively localized to the plasma membrane (see below) and to small vesicular structures in primary *pallid* melanocytes (Figure 2d) and in immortalized melan-mu (Figure 3f), melan-pa (see Figure 8)

Figure 2. Typr1 is mislocalized to syntaxin 13-positive endosomes in BLOC-1-deficient primary melanocytes. IFM analyses of primary melanocytes from wild-type C57BL/6J (a–c) or *pallid* (d–i) mice that were untreated (d–f) or infected with recombinant retroviruses expressing PaHA (g–i) and double labeled for Typr1 (a, d, and g) and syntaxin 13 (b, e, and h); merged images are shown in c, f, and i. Arrowheads and arrows indicate puncta labeling for Typr1 and syntaxin 13, respectively; in *pallid* cells, the same puncta label for both. Bars, 10 μ m.

and melan-rp cells (our unpublished data). These structures did not label for markers of late endosomes (Supplemental Figure S2), but they largely overlapped ($66 \pm 14\%$ of peripheral puncta in *pallid* cells compared with $14 \pm 7\%$ in control C57BL/6J cells) with the early endosomal tSNARE syn13 (Figures 2f and 3i). On restoring the missing BLOC-1 subunit transiently (*pallid*:PaHA) or stably (melan-mu:MuHA, melan-rp:BLOS3, and melan-pa:PaHA) by retroviral transduction, Typr1 localization to pigmented melanosomes was restored (Figures 2g and 3k; our unpublished data) and overlap in the cell periphery with syn13 was reduced (Figures 2i and 3n; overlap near the Golgi likely reflects the high concentration of organelles near the microtubule-organizing center). Syn13 distribution was not itself affected by BLOC-1 deficiency, as judged by the similar degree of proximity to EEA1 (Figure 3, e, j, and o) and to AP-3 (Supplemental Figure S1) in all cells. These data suggest that Typr1 is selectively mislocalized to syn13-containing early endosomes in the absence of BLOC-1.

To extend these results, cells were analyzed by immunoelectron microscopy (IEM). Syn13 could not be detected reproducibly by IEM, so early endosomes were labeled by Tf-FITC that was internalized for 15 min before fixation, and ultrathin cryosections were immunogold labeled for Typr1 alone or with anti-FITC. Labeling for Typr1 in melan-mu cells was predominantly observed on the limiting membrane of enlarged vacuolar endosomes that also labeled for internalized Tf-FITC and contained a few internal vesicles (Figure 4, a and c, and Table 1); sparse Typr1 labeling was sometimes observed on the internal membranes (Figure 4c). Typr1 labeling was also enriched in tubular endosomes, defined as Tf-FITC-containing tubulovesicular elements ap-

posed or continuous with the vacuolar domain (Figure 4c) or near the Golgi (Figure 4d), and also at the plasma membrane (Figure 4e), often in clathrin-coated invaginations (Figure 4e, inset). Typr1 labeling was detected only sparsely in late endosomal multivesicular bodies (Figure 4a) and was largely excluded from striated, elongated compartments with hallmarks of nonpigmented stage II melanosomes (Figure 5b and Table 1), excluding the possibility that Typr1 is targeted to melanosomes that aberrantly lack pigment. The latter were densely immunogold labeled for Pmel17 (Figure 5c), a protein normally enriched in stage II melanosomes. A similar pattern of Typr1 localization was observed in primary *pallid* melanocytes (Figure 5a), although labeling overall was less dense and more concentrated in tubulovesicular than in vacuolar structures. By contrast, Typr1 in “rescued” melan-mu:MuHA cells (Figure 4b and Table 1), like in immortalized “wild-type” melan-a cells (Supplemental Figure S3, a and inset) and primary C57BL/6J melanocytes (our unpublished data), was detected predominantly on the limiting membrane of stage III and IV melanosomes and to adjacent tubular membranes, with much less pronounced labeling of Tf-FITC-positive endosomes near the Golgi and vacuolar endosomes (Table 1). The data thus confirm that in the absence of BLOC-1, Typr1 is largely trapped in early endosomes and excluded from melanosomes, and they suggest that BLOC-1 might facilitate removal of Typr1 from early endosomes toward melanosomes. This is consistent with the steady-state localization of BLOC-1 to Tf-accessible tubulovesicular early endosomes in melanocytic cells (Di Pietro *et al.*, 2006) and of reconstituted BLOC-1 in melan-mu:MuHA cells to similar tubulovesicular structures near

Figure 3. Tyrp1 is mislocalized to syntaxin 13-positive endosomes in melan-mu cells. IFM analyses of immortal wild-type melan-a (a–e), BLOC-1-deficient melan-mu (f–j), or rescued melan-mu:MuHA cells (k–o), by using antibodies to Tyrp1 (a, f, and k), syn13 (b, g, and l), and EEA1 (c, h, and m). Merged images are shown for syn13 with Tyrp1 (d, i, and n) or with EEA1 (e, j, and o). Arrows and arrowheads indicate puncta labeling for Tyrp1 and syn13, respectively; in melan-mu, the same puncta label for both. Bar, 10 μ m.

the Golgi, melanosomes, and endosomal vacuoles (Supplemental Figure S3, b and c).

Interestingly, a cohort of tyrosinase—the enzyme that mediates the initial steps of melanin synthesis—was also aberrantly enriched in vacuolar early endosomes together with Tyrp1 and in multivesicular late endosomes in BLOC-1-deficient cells (Figure 5d and Table 1), but a large cohort was correctly localized to the nonpigmented melanosomes (Figure 5c and Table 1). Thus, tyrosinase trafficking to melanosomes is only partially dependent on BLOC-1. This is con-

sistent with two known tyrosinase trafficking pathways (Theos *et al.*, 2005) and indicates that the BLOC-1 transport defect to melanosomes is cargo selective.

BLOC-1 Prevents Biosynthetic Delivery of Tyrp1 to the Cell Surface

Tyrp1 was enriched at the surface of BLOC-1-deficient melanocytes relative to controls, as shown by IFM analysis of primary melanocytes labeled for Tyrp1 before fixation (Figure 6a) and quantitated by flow cytometry of melan-mu and

Figure 4. Tyrp1 accumulates in expanded vacuolar early endosomes in BLOC-1-deficient cells. IEM analyses of ultrathin cryosections of melan-mu (a and c–e) and melan-mu:MuHA (b). Cells were incubated with Tf-FITC for 15 min at 37°C to fill early endosomes before fixation. Sections were immunogold labeled with anti-Tyrp1 (PAG10) and anti-FITC (PAG15). In melan-mu, both Tyrp1 and Tf-FITC accumulate in vacuolar endosomes (End., expanded in size and number relative to melan-mu:MuHA; a and c), in tubulovesicular structures (arrows) emanating from the vacuolar endosomes (c) or near the Golgi (GA; d), and on the plasma membrane (PM; e), but not in MVBs (a). (e) Inset, Tyrp1 and Tf-FITC in a coated pit at the plasma membrane. In melan-mu:MuHA, Tyrp1 accumulates on stage IV melanosomes (IV) and endosomal tubules that closely appose melanosomes (b). Bar, 200 nm.

Table 1. Quantification of Tyrp1 and tyrosinase immunogold labeling in melan-mu and melan-mu:MuHA

	Golgi	TGN	Vacuolar endosomes	TVEs/End.	MVBs	Mels.	TVEs/Mel.	Lysosomes	Vesicles
Tyrp1									
melan-mu: MuHA	13.0	3.4	6.2	5.8	1.9	61.0	7.2	1.3	0.2
melan-mu	4.6	5.5	50.8	21.0	8.3	1.5	0.6	0.1	7.7
Tyrosinase									
melan-mu: MuHA	1.2	7.0	10.8	7.6	6.8	60.7	2.3	0.7	3.0
melan-mu	6.2	6.1	18.7	6.0	24.8	33.8	1.6	0.5	2.3

Percentage of total counted immunogold particles detected in each compartment, excluding the cell surface, from 15 cell profiles each. Values based on the following total number of gold particles: Tyrp1 in melan-mu:MuHA, 2382 particles; Tyrp1 in melan-mu, 1502 particles; tyrosinase in melan-mu:MuHA, 2287 particles; and tyrosinase in melan-mu, 1898 particles. Endosomes were defined by labeling for Tf-FITC after 15 min of uptake. TVEs, tubulovesicular endosomes near vacuolar endosomes or the Golgi (TVEs/End.) or near melanosomes (TVEs/Mel.). MVBs, both internal and limiting membrane of multivesicular bodies. Mels, melanosome stages II, III, and IV; in melan-mu melanosomes are defined as compartments that resemble stage II and that label for Pmel17. Compartments with the highest percentage within each cell line are indicated in bold.

Figure 5. Tyrosinase and Pmel17, but not Tyrp1, localize to striated melanosomes in BLOC-1-deficient cells. (a) Ultrathin cryosections of *pallid* melanocytes were immunogold labeled for Tyrp1 (PAG10). Note the labeling of tubulovesicular structures near the Golgi (GA; a) and vacuolar endosomes (End, inset). (b–d) Ultrathin cryosections of melan-mu cells were immunogold labeled for Tyrp1 (PAG15; b), for Pmel17 (PAG15) and tyrosinase (Tyr, PAG10; c), or for Tyrp1 (PAG15) and tyrosinase (Tyr, PAG10; d). The image shown in b was taken from a set of labeled sections in which dense labeling was observed on endosomes (data not shown). Note the absence of labeling for Tyrp1 over elongated, striated structures with features of stage II melanosomes (II; b) that densely label for Pmel17 and tyrosinase (c and d). Tyrosinase is also detected in vacuolar endosomes (End) that contain Tyrp1 (d). Bars, 0.2 μ m.

melan-rp cells relative to BLOC-1-reconstituted cells (Figure 6b; the more modest Tyrp1 surface expression in melan-rp relative to melan-mu likely reflects residual BLOC-1 activity in *rp* cells; Starcevic and Dell'Angelica, 2004; our unpublished data). Nevertheless, BLOC-1-deficient cells had lower total cellular Tyrp1 levels ($\sim 60\%$ that of BLOC-1-reconstituted cells; Figure 6c), indicating that the increased surface levels reflect Tyrp1 missorting and not increased expression; the decreased Tyrp1 cellular levels in these immortalized BLOC-1-deficient melanocytes may reflect dysregulated expression, because Tyrp1 half-life, measured by metabolic pulse/chase analysis, was unchanged compared with controls (our unpublished data). Moreover, the 7- and fourfold increases in surface expression in melan-mu and melan-rp cells, respectively, were specific to Tyrp1, because surface levels of another melanosomal protein (Pmel17) or the lysosomal protein LAMP-1 were unchanged or increased up to only twofold, respectively, relative to the corresponding BLOC-1-reconstituted cells (Figure 6b; tyrosinase levels could not be quantitated because the antibody recognizes only fixed or denatured protein). Tf receptor (TfR) surface levels were also dramatically increased but paralleled by an as yet unexplained increase in total cellular levels in BLOC-1-deficient cells (Figure 6, b–d) and thus were not attributed to missorting (Figure 7, c and f). Lower TfR levels in rescued cells reflected BLOC-1 recovery and not an artifact of transgene transduction, because stably transduced melan-mu cells expressing PaHA retained high cellular TfR levels (unpublished data). Comparison of the relative ratio of surface to total cellular levels shows a specific increase for Tyrp1,

but not for TfR, in BLOC-1-deficient cells relative to BLOC-1-reconstituted cells (Figure 6d). Thus, Tyrp1 is specifically redistributed to the cell surface upon BLOC-1 depletion.

To determine whether altered endosomal dynamics explain the increased Tyrp1 surface expression in BLOC-1-deficient cells, we compared internalization and endocytic recycling rates for several proteins in BLOC-1-deficient and reconstituted cells by using quantitative flow cytometry. The rate of Tyrp1 endocytosis was reduced 1.7-fold in melan-mu cells (Figure 7a; 23%/min versus 39%/min for melan-mu: MuHA) and 1.3-fold in melan-rp (our unpublished data) relative to rescued cells. These reductions in endocytic rate cannot explain the respective seven- and fourfold increases in Tyrp1 surface levels for several reasons. First, given the increased surface expression in melan-mu, a twofold decrease in endocytic rate represents a 3.5-fold increase in flux of internalized Tyrp1 (see below). Second, a similar decrease in endocytic rate was observed for LAMP-1 (Figure 7b; 37%/min for melan-mu:MuHA, 15%/min for melan-mu), corresponding well with the twofold increase in surface LAMP-1. Third, both the fraction and rate of endocytosed Tyrp1 undergoing recycling to the cell surface were also specifically decreased twofold in melan-mu cells (Figure 7e; 5.1%/min for melan-mu:MuHA, 2.6%/min for melan-mu; TfR recycling was similar in both cells—6.4%/min—as shown in Figure 7f). The reduced rate and fraction of Tyrp1 recycling, nevertheless reflecting increased recycling flux due to the high starting values, should negate the effects of the decreased endocytic rate on surface Tyrp1 levels. Thus, effects on endocytosis or endocytic recycling alone do not

Figure 6. Accumulation of Tyrp1 at the cell surface of BLOC-1-deficient cells. (a) Surface IFM analyses of primary melanocytes from C57BL/6J (left) or *pallid* (right) mice. Cells on ice were labeled with Alexa-488-conjugated anti-Tyrp1 antibody and then washed and fixed. (b) Flow cytometry analysis of BLOC-1-deficient (melan-mu and melan-rp) and rescued (melan-mu:MuHA and melan-rp:BLOS3) cells for surface levels of Tyrp1, TfR, LAMP-1, and Pmel17 by using unlabeled primary antibodies and Alexa-488-conjugated secondary antibody. Relative MFI was normalized to 1.00 for melan-mu:MuHA or melan-rp:BLOS3, respectively, within each experiment. Values represent mean and SD over two (melan-rp, melan-rp:BLOS3) or five (melan-mu, melan-mu:MuHA) experiments, each performed in duplicate or quadruplicate. (c) SDS-PAGE fractionated whole cell lysates of melan-mu, melan-mu:MuHA, melan-rp, and melan-rp:BLOS3 cells were immunoblotted with rabbit anti-Tyrp1 or anti-TfR. Each blot was reprobed with anti-tubulin as a loading control. (d) Band intensities of TfR or Tyrp1 from equal cell numbers in immunoblotting experiments (like in c) were quantified and normalized relative to respective rescued cell values. Data represent the average of two to three experiments, except asterisk (*), which represents only one experiment in which the range of detection was linear (does not include the panel shown in c). The normalized total cellular expression value was then divided into the normalized surface expression value from b to define relative surface: total expression of Tyrp1 and TfR.

account for the specific increase in Tyrp1 surface levels in BLOC-1-deficient cells.

If BLOC-1 were to facilitate biosynthetic Tyrp1 trafficking to melanosomes through endosomes, then BLOC-1 deficiency might increase Tyrp1 flux through the cell surface by “default” as a consequence of failing to withdraw Tyrp1 from endosomes. To monitor cell surface flux, we measured the rate of antibody uptake in cells exposed continuously to Alexa-488-conjugated anti-Tyrp1 antibody. As predicted, melan-mu cells accumulated significantly more anti-Tyrp1

antibody than melan-mu:MuHA, reaching 15-fold higher levels by 2 h (Figure 7g). To limit the analysis primarily to biosynthetic Tyrp1 delivery, the experiment was repeated after preincubation with excess unlabeled anti-Tyrp1 antibody for 20 min, enough time to prebind most of the cell surface and endosomal recycling Tyrp1 pools (Figure 7e) and thus largely exclude them from the analysis. Preincubation dramatically reduced the accumulation of labeled antibody (Figure 7h), indicating that most of the cell surface flux of Tyrp1 reflects recycling from an endosomal pool. However, even after the preincubation, nearly fivefold more labeled antibody accumulated by 2 h in melan-mu than melan-mu:MuHA cells (Figure 7h, inset). Moreover, continuous antibody uptake after 20 min was dramatically inhibited by treatment of melan-mu, but not melan-mu:MuHA, with brefeldin A at a concentration sufficient to disrupt the Golgi complex (Figure 7i; in control experiments not shown, brefeldin A did not block Tyrp1 recycling). Together, these results indicate that biosynthetic Tyrp1 delivery to the cell surface is significantly increased in melan-mu cells. Given the balanced decrease in endocytic and recycling rates, biosynthetic delivery must account for the increased endosomal flux of Tyrp1 in BLOC-1-deficient cells. Moreover, the failure of brefeldin A to affect Tyrp1 cell surface flux in melan-mu:MuHA cells suggests that most surface Tyrp1 does not normally derive from biosynthetic delivery. Together, the data suggest that BLOC-1 facilitates diversion of biosynthetically delivered Tyrp1 out of endosomes and toward melanosomes.

BLOC-1 Deficiency Likely Reduces Endocytic Rates Indirectly by Increasing Endocytic Flux

How might the decreased endocytic rate for Tyrp1 and LAMP-1 in BLOC-1-deficient cells be explained? Interestingly, the endocytic rate for the overexpressed TfR in melan-mu cells was similarly decreased (Figure 7c; 39%/min for melan-mu:MuHA, 15%/min for melan-mu), whereas that of Pmel17 was not (Figure 7d). Endocytosis of LAMP-1 and TfR are mediated by cytoplasmic tyrosine-based signals (YxxØ class, where Ø represents a bulky hydrophobic amino acid and x is any amino acid) that bind to the μ subunits of AP-2 and related adaptors (Bonifacino and Traub, 2003). By contrast, Pmel17 endocytosis is mediated by a dileucine-based (D/ExxxLL class) signal (Theos *et al.*, 2006), similar to those that engage different sites on adaptors (Bonifacino and Traub, 2003; Janvier *et al.*, 2003) (Figure 8a). Endocytosis mediated by both signal classes is independently saturable (Marks *et al.*, 1996). The Tyrp1 cytoplasmic domain contains both YxxØ and dileucine consensus motifs (Vijayasaradhi *et al.*, 1995); thus, decreased endocytic rates in BLOC-1-deficient cells might reflect saturation of YxxØ- but not dileucine-dependent internalization due to increased surface expression of Tyrp1 and other proteins (such as TfR). Consistently, purposeful overexpression of Tyrp1 by transfection in wild-type 1011-mel melanocytic cells increased the surface expression of LAMP-1 (Figure 8, b–e), but not Pmel17 (Figure 8, h–k). Mutagenesis of both Tyrp1 consensus sorting signals ablated this effect (Figure 8, f, g, l, and m). Thus, decreased endocytosis is likely a consequence, not a cause, of the increased flux of Tyrp1, TfR, and other proteins through the surface of BLOC-1-deficient cells. A similar phenomenon may account for increased Tyrp1 surface expression in AP-3- and/or BLOC-2-deficient cells (our unpublished data).

surface flux of Tyrp1. (g) Cells were incubated at 37°C with saturating Alexa-488-conjugated anti-Tyrp1 antibody for the indicated times. Total accumulated fluorescence was quantified by flow cytometry. (h) Same as g, except that cells were preincubated for 20 min at 37°C with excess unlabeled anti-Tyrp1 before exposure to fluorochrome-conjugated antibody. The main panel uses the same scale as g; inset, same data plotted on a different scale to emphasize differences between the cell types. (i) Same as g, except that indicated cells were treated with 10 μ g/ml brefeldin A for 1 h before and throughout the experiment. All values are the mean and SD from two separate experiments performed in duplicate or triplicate. Melan-mu and Melan-mu:MuHA are abbreviated mu and mu:MuHA, respectively, in panels h and i.

BLOC-2, but Not AP-3, Acts Downstream of BLOC-1 in Tyrp1 Transport from Endosomes to Melanosomes

BLOC-1 interacts physically with BLOC-2 and AP-3, and surface flux of Tyrp1 is increased in BLOC-2- and AP-3-deficient melanocytes (Di Pietro *et al.*, 2006). To establish functional relationships between BLOC-1, BLOC-2, and AP-3 in Tyrp1 trafficking, intracellular Tyrp1 localization was assessed relative to various markers in BLOC-1-deficient melan-pa, BLOC-2-deficient melan-coa (lacking the HPS3 subunit; Suzuki *et al.*, 2001; Gautam *et al.*, 2004), and AP-3-deficient melan-pe cells (lacking the β 3A subunit; Theos *et al.*, 2005) derived from *pallid*, *cocoa*, and *pearl* mice, respectively. Unlike in BLOC-1-deficient cells, pigmented melanosomes are detectable in melan-coa and melan-pe by light microscopy (Supplemental Figure S4, a–d), although they are not as dense as in control cells. As in melan-mu (Figure 3), Tyrp1 in melan-pa localized to discrete puncta that overlapped largely with syn13 in the cell periphery ($44 \pm 11\%$, compared with $12 \pm 5\%$ for reconstituted melan-pa:PaHA cells; Figure 9, a–c). In melan-coa, Tyrp1 distributed in larger, more discrete puncta that overlapped less ($28 \pm 7\%$) with syn13 (Figure 9, d and e), minimally ($<10\%$) with the modestly pigmented melanosomes (Figure 9, d and f), and not at all with markers of late endosomes/lysosomes or stage II melanosomes (Supplemental Figure S4, e–g, k–m). By IEM, Tyrp1 localized primarily to tubulovesicular structures, only few of which labeled weakly for internalized Tf-FITC (Figure 9j and Supplemental Figure S5); many of these structures were closely apposed to or continuous with multivesicular bodies (MVBs) (Figure 9k and Supplemental Figure S5), suggesting that they are endosomal domains from which Tyrp1 may be delivered to late endosomes. Consistently, immunogold labeling density for Tyrp1

was lower in melan-coa than in the other cells analyzed, perhaps reflecting Tyrp1 degradation in late endosomes or lysosomes. These data suggest that BLOC-2 facilitates Tyrp1 trafficking to melanosomes from an endosomal domain that partially overlaps with but is largely distinct and downstream from that of BLOC-1.

In AP-3-deficient melan-pe cells, intracellular Tyrp1 localized nearly exclusively to pigmented melanosomes by IFM (Figure 9, g–i), despite high Tyrp1 surface expression (our unpublished data); this was confirmed by IEM, in which Tyrp1 colocalized extensively in pigmented stage IV melanosomes with tyrosinase (Supplemental Figure S5, c). As expected, very little overlap was observed with markers of stage II melanosomes, late endosomes, or lysosomes (Supplemental Figure S4, h–j, n–p). Intriguingly, syn13 was re-distributed to melanosomes in melan-pe cells (Figure 9, g–i). This suggests that AP-3 does not function directly in anterograde Tyrp1 transport but that it may regulate syn13 cycling, perhaps by retrieval from melanosomes.

DISCUSSION

The genes encoding five of the eight known BLOC-1 subunits are mutated in mouse and human HPS variants, and polymorphisms in the dysbindin subunit gene are associated with schizophrenia, but the function of BLOC-1 has remained elusive. Our data show that BLOC-1 functions in a protein transport pathway from early endosomes to maturing melanosomes and that this pathway is obligatory for at least one melanosomal cargo protein, Tyrp1. The HPS-associated protein complex BLOC-2 seems to function downstream of BLOC-1 in this pathway, but AP-3 seems to regulate different cargo. Our data have important implications

a. Protein : Cytoplasmic domains

hTyrp1 : TM-RARRSMDEANQPLLTDQYQCYAEYEKLQNPNSV
 hLAMP-1: TM-RKRSHAGYQTI
 hPmel17 : TM-25 aa-PRIFCSCPIGENSPLLSGQQV
 mTfR : 9 aa-SNLFGEPLSYTRFSLARQVD-35 aa-TM

Figure 8. Increased surface expression of Tyrp1 induces increased surface expression of LAMP-1 but not Pmel17. (a) Sequences of cytoplasmic domains of human (h) Tyrp1, LAMP-1, and Pmel17 and mouse (m) TfR. Position of the transmembrane domain (TM) and distance in amino acid residues (aa) from the indicated sequence are shown. Relevant sorting signal motifs are underlined. (b–m) 1011-mel human melanoma cells were transiently transfected with high levels of vector alone (b, c, h, and i), Tyrp1 wild-type (WT; d, e, j, and k), or Tyrp1 with alanine substitutions for tyrosine and leucine residues within the YxxØ and LL motifs (LLY; f, g, l, and m). Cells were analyzed by flow cytometry for surface Tyrp1 and LAMP-1 (b–g) or Tyrp1 and Pmel17 (h–m) after staining with directly conjugated primary antibodies. b, d, and f and h, j, and l, two-dimensional dot plots showing labeling of individual cells with each antibody. c, e, and g and i, k, and m, single color analysis of LAMP-1 (c, e, and g) or Pmel17 (i, k, and m) labeling for all cells (c and i) or for gated cells that do (black line; e, g, k, and m) or do not (filled curve; e, g, k, and m) express Tyrp1 above threshold. RC counts, relative cell counts. Representative of two experiments in 1011-mel cells, each in duplicate, and four experiments in HeLa cells.

for generalized endosomal transport, LRO biogenesis, and the etiology of HPS.

Previous evidence has demonstrated selective endosomal protein sorting defects in AP-3-deficient melanocytes (Huizing *et al.*, 2001; Theos *et al.*, 2005), altered distribution of selected melanosomal cargo in BLOC-3- and BLOC-2-deficient human melanocytes (Boissy *et al.*, 2005; Richmond *et al.*, 2005), and enhanced cell surface flux of Tyrp1 in mouse

melanocytes lacking AP-3, BLOC-1, or BLOC-2 (Di Pietro *et al.*, 2006). We now show for the first time (to our knowledge) that BLOC-1-deficient melanocytes misroute selected cargo destined for melanosomes from early endosomes. Our data further suggest that functional loss of BLOC-2 interferes with Tyrp1 delivery to melanosomes from an endosomal intermediate that is at least partially downstream of BLOC-1, with likely “default” targeting to late endosomes/lysosomes and consequent degradation as suggested in primary BLOC-2-deficient melanocytes (Di Pietro *et al.*, 2006). At least two other protein complexes affected by mouse HPS models—HOPS, for which the Vps33a subunit is deficient in *buff* mice (Suzuki *et al.*, 2003), and rab geranylgeranyl transferase II, for which the α subunit is deficient in *gunmetal* mice (Detter *et al.*, 2000)—are also thought to impact early endosomal dynamics (Li *et al.*, 2004; Richardson *et al.*, 2004), and Rab38, mutated in a rat HPS model, regulates melanosomal transport through what are likely endosomal intermediates (Wasmeier *et al.*, 2006). Thus, most forms of HPS reflect defective transport from early endosomes to selected LROs. These findings highlight the importance of early endosomes as intermediates in post-Golgi transport to LROs and the adaptability of the endosomal system in specialized cell types such as melanocytes.

Whereas both AP-3 and BLOC-1 function in sorting to melanosomes from endosomes, they regulate at least partially distinct cargoes. Although both complexes localize at steady state in melanocytic cells to tubular early endosomes, BLOC-1 is found on uncoated tubulovesicular domains distinct from the AP-3/clathrin-coated buds that concentrate tyrosinase (Theos *et al.*, 2005; Di Pietro *et al.*, 2006). Moreover, AP-3 deficiency dramatically alters intracellular tyrosinase distribution but only modestly affects that of Tyrp1 (Figure 9; Huizing *et al.*, 2001; Theos *et al.*, 2005), consistent with the differential binding of AP-3 σ_3/δ hemicomplexes to the tyrosinase and Tyrp1 dileucine-based sorting signals (Theos *et al.*, 2005). By contrast, the inhibition of melanosome transport in BLOC-1-deficient cells is only partial for tyrosinase but complete for Tyrp1. The severe hypopigmentation of BLOC-1-deficient cells contrasts with the brown pigmentation characteristic of Tyrp1 deficiency (Bennett *et al.*, 1989; Boissy *et al.*, 1996) and indicates that additional critical cargoes, besides Tyrp1, absolutely require BLOC-1 for melanosome transport. These additional cargoes do not likely require AP-3 to access melanosomes, because AP-3-deficient melanocytes are highly pigmented (Theos *et al.*, 2005). Together, the data suggest that AP-3- and BLOC-1 regulate melanosome delivery of distinct cargoes through distinct early endosomal intermediates. That BLOC-1 and AP-3 regulate at least partially distinct cargo transport pathways is consistent with the observed synthetic effects of BLOC-1 and AP-3 deficiency on the structure and function of several LROs (Di Pietro *et al.*, 2006; Gautam *et al.*, 2006). We speculate that the copurification (Salazar *et al.*, 2006) and physical interaction (Di Pietro *et al.*, 2006) of AP-3 and BLOC-1 reflect a role for both complexes in regulating the trafficking of different classes of cargoes such as SNAREs, consistent with the mislocalization of syn13 to melanosomes in AP-3-deficient melanocytes (Figure 9).

Whether BLOC-1 interacts directly with its cargo or facilitates downstream membrane dynamics is not known. Although the primary endosomal sorting defect in BLOC-1-deficient melanocytes is cargo specific, the cells also exhibit a generalized decrease in the rate of clathrin-dependent endocytosis mediated by YxxØ but not dileucine motifs. Nevertheless, the endocytic flux for affected cargoes is significantly increased owing to the increased cell surface ex-

Figure 9. Typr1 is mislocalized to a distinct endosomal subdomain in cells lacking BLOC-2, but not cells lacking AP-3. (a–i) IFM analyses of melan-pa, melan-coa, and melan-pe cells that were double labeled for Typr1 (red; a, d, and g) and syn13 (green; b, e, and h); corresponding bright field (BF) images are shown in c, f, and i. Insets, 2.5×-magnified overlays of the boxed regions comparing syn13 to Typr1 (left), syn13 to melanosomes (middle), and Typr1 to melanosomes (right); melanosomes are pseudocolored blue from an inverted bright field image. Arrows point to the same puncta in all three fields. (j and k) IEM analyses of melan-coa cells immunogold labeled for Typr1 (PAG10) with (j) or without (k) internalized Tf-FITC (15 min; PAG15). Arrows, Typr1 labeling; arrowhead, Tf-FITC. II and IV, stage II and IV melanosomes. End, vacuolar endosome. Asterisk (*), pigmented structure with features of an MVB. Note the MVBs nearby Typr1-containing endosomes.

pression of Typr1, TfR, and likely other cargoes. As shown previously (Marks *et al.*, 1996; Warren *et al.*, 1997) and corroborated here, increased flux can saturate the endocytic machinery, resulting in reduced endocytic rates for proteins with related endocytic signals. Thus, decreased endocytic rates for Typr1 and other cargoes are most likely an indirect effect of the increased exocytic flux and/or expression of specific endocytic cargo induced by BLOC-1 deficiency. These results imply that defects in endosomal transport machinery can indirectly redistribute cargo to the cell surface, perhaps partially explaining the enhanced surface expression of several proteins in AP-3- and BLOC-2-deficient cells (Dell'Angelica *et al.*, 1999; Le Borgne *et al.*, 2001; Di Pietro *et al.*, 2006; Salazar *et al.*, 2006).

How does BLOC-1 facilitate trafficking from endosomes to melanosomes? The paucity of newly synthesized Typr1 that transits the plasma membrane in wild-type melanocytes suggests that Typr1 is normally routed directly from the trans-Golgi network (TGN) to endosomes and then to melanosomes (Figure 7i); indeed, this is corroborated by interfer-

ing with endosomal function in wild-type melanocytes (our unpublished data). The high flux of newly synthesized Typr1 through both the cell surface and early endosomes in BLOC-1-deficient cells is thus consistent with either of two models for BLOC-1 function. In the first model, BLOC-1 promotes fusion of TGN-derived Typr1-containing vesicles with early endosomes, such that these vesicles are mistargeted to the plasma membrane when BLOC-1 is absent. However, the high content of endosomal Typr1 and complete absence of Typr1 from melanosomes in these cells seems inconsistent with such a model, particularly given the localization of Typr1 to a different endosomal domain in BLOC-2-deficient cells or to melanosomes in AP-3-deficient cells, despite similar levels of cell surface—and hence internalized—Typr1. We thus favor a second model (Figure 10), in which BLOC-1 promotes the exit of cargo from early endosomal membranes toward melanosomes. Increased surface delivery and endosomal content of Typr1 would thus reflect futile cycles of endocytosis and recycling (of both internalized and biosynthetically derived cargo) resulting

Figure 10. Model for BLOC-1 and BLOC-2 function in Tyrp1 trafficking from endosomes to melanosomes. BLOC-1 facilitates Tyrp1 exit from an early endosome subdomain toward an intermediate compartment, from which BLOC-2 facilitates targeting to melanosomes. Arrows represent vesicular or tubular traffic between compartments; thick arrows represent the major pathway in wild-type cells, and dashed arrows represent pathways taken in BLOC-1-deficient cells.

from the inability to “drain” endosomes toward melanosomes. This model best explains the complete absence of Tyrp1 from melanosomes, the accumulation of vacuolar endosomes, and the inefficient Tyrp1 recycling in BLOC-1-deficient cells. The accumulation of Tyrp1 in a partially distinct and likely downstream compartment in BLOC-2-deficient cells would favor a model in which the BLOC-1-dependent early endosome-derived membranes pass through another endosomal intermediate, from which BLOC-2 facilitates fusion with melanosomes (Figure 10); that this intermediate is contiguous with early endosomes is suggested by the partial accessibility of BLOC-2-labeled tubulovesicular structures in MNT-1 melanoma cells by internalized Tf (Di Pietro *et al.*, 2006). This may be the same intermediate from which Rab38 regulates Tyrp1 and tyrosinase delivery to melanosomes (Wasmeier *et al.*, 2006). We speculate that the interaction of BLOC-1 with syn13 (Huang *et al.*, 1999; Moriyama and Bonifacio, 2002) and SNAP-23/25 (Ilardi *et al.*, 1999; Buxton *et al.*, 2003) might regulate the formation of these intermediates and/or their ability to fuse with melanosomes.

BLOC-1 subunits are ubiquitously expressed, but overt phenotypes of BLOC-1 deficiency, as evidenced by HPS, are only observed in certain cell types (Bonifacio, 2004; Di Pietro and Dell’Angelica, 2005). It is thus likely that the BLOC-1-dependent pathway that we have defined is ubiquitous in mammals, but it has been exploited by specialized cell types for delivery of specific cargo to LROs, such as melanosomes, platelet dense granules, and lung lamellar bodies, and/or to synaptic vesicles (McGarry *et al.*, 2002; Talbot *et al.*, 2004; Bray *et al.*, 2005; Guttentag *et al.*, 2005). Our data suggest that at least one critical cargo protein is targeted from early endosomes to each of these organelles in a BLOC-1-dependent manner.

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