Redundant Roles of BIG2 and BIG1, Guanine-Nucleotide Exchange Factors for ADP-Ribosylation Factors in Membrane Traffic between the *trans*-Golgi Network and Endosomes

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BIG2 and BIG1 are closely related guanine-nucleotide exchange factors (GEFs) for ADP-ribosylation factors (ARFs) and are involved in the regulation of membrane traffic through activating ARFs and recruiting coat protein complexes, such as the COPI complex and the AP-1 clathrin adaptor complex. Although both ARF-GEFs are associated mainly with the *trans*-Golgi network (TGN) and BIG2 is also associated with recycling endosomes, it is unclear whether BIG2 and BIG1 share some roles in membrane traffic. We here show that knockdown of both BIG2 and BIG1 by RNAi causes mislocalization of a subset of proteins associated with the TGN and recycling endosomes and blocks retrograde transport of furin from late endosomes to the TGN. Similar mislocalization and protein transport block, including furin, were observed in cells depleted of AP-1. Taken together with previous reports, these observations indicate that BIG2 and BIG1 play redundant roles in trafficking between the TGN and endosomes that involves the AP-1 complex.

INTRODUCTION

Membrane traffic between subcellular compartments is mediated by vesicular and tubular intermediates. Their formation from donor compartments is regulated by a variety of cytosolic proteins, including small GTPases and coat proteins (Kirchhausen, 2000). ADP-ribosylation factors (ARFs) are a family of small GTPases that trigger budding of coated carrier vesicles by recruiting coat protein complexes onto donor membranes. Protein complexes that are recruited by ARFs include the COPI complex onto the *cis*-Golgi, the AP-1 clathrin adaptor complex onto the *trans*-Golgi network (TGN) and endosomes, the AP-3 complex onto endosomes, and the monomeric GGA proteins onto the TGN.

ARFs cycle between an inactive GDP-bound state and an active GTP-bound state during which they interact with coat proteins and other effectors. Exchange of bound GDP for GTP on ARFs is stimulated by a family of guanine nucleotide exchange factors (GEFs) that contain a Sec7 catalytic domain, whereas intrinsic GTPase activity of ARFs is enhanced by a family of GTPase-activating proteins (Donaldson and Jackson, 2000; Jackson and Casanova, 2000; Shin and Nakayama, 2004; Nie and Randazzo, 2006). The ARF-GEFs are classified into several groups including the high-

molecular-weight GEFs of the Gea/GBF and Sec7/BIG groups. These are involved in the regulation of membrane traffic and are sensitive to brefeldin A (BFA), which inhibits various trafficking processes and causes deformation of the Golgi apparatus and recycling endosomes (Jackson, 2000; Jackson and Casanova, 2000; Shin and Nakayama, 2004).

GBF1 is a sole member of the mammalian Gea/GBF group and functions primarily in the trafficking between the *cis*-Golgi and the ER-Golgi intermediate compartment (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Zhao *et al.*, 2002; García-Mata *et al.*, 2003; Zhao *et al.*, 2006). On the other hand, BIG2 and BIG1 of the Sec7/BIG group (Morinaga *et al.*, 1996, 1997; Mansour *et al.*, 1999; Togawa *et al.*, 1999) show considerable similarity to each other in their primary sequence and domain organization (Mouratou *et al.*, 2005). Although early studies showed that both BIG2 and BIG1 are associated mainly with the TGN (Mansour *et al.*, 1999; Yamaji *et al.*, 2000; Shinotsuka *et al.*, 2002a,b; Zhao *et al.*, 2002), we and others later showed that BIG2 is also associated with recycling endosomes, where it recruits the AP-1 complex through activating ARFs and regulates trafficking of cargo proteins through these compartments (Shin *et al.*, 2004; Shen *et al.*, 2006). However, it is currently not clear if BIG2 and BIG1 play distinct roles or share some roles. In the present study, we exploit an RNA interference (RNAi) approach and reveal that knockdown of BIG2 alone affects localization of proteins associated with recycling endosomes; knockdown of BIG1 alone shows no obvious effects on organelle markers; and knockdown of both BIG2 and BIG1 causes delocalization of various proteins that reside in the TGN and recycling endosomes and blocks retrograde trafficking of furin from late endosomes to the TGN.

MATERIALS AND METHODS

Antibodies, Reagents, and Plasmids

Preparation and affinity purification of polyclonal rabbit antibodies against BIG1 and BIG2 were described previously (Ishizaki *et al.*, 2006). Monoclonal

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Abbreviations used: ARF, ADP-ribosylation factor; HRP, horseradish peroxidase; GEF, guanine nucleotide exchange factor; PACS, phosphofurin acidic cluster sorting protein; TGN, *trans*-Golgi network; TfR, transferrin receptor; VSVG, vesicular stomatitis virus G protein.

mouse antibodies against CD4, EEA1, GM130, golgin-245, GGA3, and γ -adaptin (Clone 88) were purchased from BD Biosciences (San Jose, CA); polyclonal rabbit antibodies against β -COP and furin from Affinity Bioreagents (Golden, CO); monoclonal mouse antibodies against γ -adaptin (Clone 100.3) and FLAG (M2) from Sigma (St. Louis, MO); monoclonal mouse anti-transferrin receptor (TfnR) from Zymed Laboratories (South San Francisco, CA); monoclonal rat anti-CD4 antibody and polyclonal sheep anti-TGN46 antibody from Serotec (Raleigh, NC); AlexaFluor-conjugated secondary antibodies and AlexaFluor488-conjugated EGF from Molecular Probes (Eugene, OR); and Cy3-conjugated and horseradish peroxidase (HRP)-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). Polyclonal rabbit anti-EEA1 antibody, monoclonal mouse anti-lysobisphosphatidic acid (LBPA) and Cy3-conjugated Shiga toxin 1 were kind gifts from Marino Zerial (MPI-CBG, Germany), Toshihide Kobayashi (RIKEN, Japan), and Naoko Morinaga (Chiba University, Japan). Construction of an expression vector for a CD4-furin fusion protein with an exoplasmic domain of CD4 and transmembrane and cytoplasmic domains of furin (see Figure 6A) was described previously (Takahashi *et al.*, 1995). Expression vectors for fluorescent protein-tagged Rab proteins were described previously (Shin *et al.*, 2004). An expression vector for FLAG-tagged TGN38 was a kind gift from Nobuhiro Nakamura (Kanazawa University, Japan).

Cell Culture, RNAi Suppression, Antibody Uptake Experiments, and Immunofluorescence Analysis

HeLa cells were cultured in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. An HeLa cell line stably expressing CD4-furin or FLAG-TGN38 was established by transfection of a pcDNA3-based expression vector followed by selection in the presence of 800 μ g/ml G418 sulfate. Knockdown of BIG2 (nucleotide residues 600-1366) or BIG1 (residues 1-988) alone or of both ARF-GEFs was performed as described previously (Ishizaki *et al.*, 2006). Knockdown of AP-1 was performed by incubating cells with a pool of small interfering RNAs (siRNAs) directed for an mRNA region of μ 1A covering nucleotide residues 95-1120 (when the A residue of the initiation Met codon is assigned as residue 1, prepared using a BLOCK-iT RNAi TOPO Transcription kit and a BLOCK-iT Dicer RNAi kit (Invitrogen, Carlsbad, CA). Indirect immunofluorescence analysis of cells cultured on coverslips was performed as described previously (Shin *et al.*, 1997, 2004; Shinotsuka *et al.*, 2002b). For staining with anti-LBPA antibody, the cells fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) were incubated with the antibody in 0.05% saponin/PBS at room temperature for 30 min.

Uptake experiments of anti-CD4 or anti-FLAG antibody by cells stably expressing CD4-furin or FLAG-TGN38 were performed as described previously (Takahashi *et al.*, 1995; Shin *et al.*, 2004, 2005) with some modifications. Briefly, HeLa cells stably expressing CD4-furin or FLAG-TGN38 were mock-treated (a pool of siRNAs for LacZ) or treated with a pool of siRNAs for BIG1 and/or BIG2 or μ 1A for 80 h and before antibody uptake were incubated with 15 mM sodium butyrate for 16 h. The cells were then incubated with monoclonal anti-CD4 (Leu3a) or anti-FLAG (M2) antibody in combination with AlexaFluor488-conjugated EGF at 19°C for 60 min, subjected to an acid wash (0.5% acetic acid, pH 3.0, 50 mM NaCl) in the case of the anti-FLAG uptake, and incubated at 37°C for the indicated periods of time. When indicated, the number of fluorescent structures of internalized anti-CD4 antibody present within the EGF-positive compartments were calculated using IPLab 4.0 software (Solution Systems, Funabashi, Japan). Briefly, punctate structures containing fluorescent EGF were set as regions of interest (ROIs) in more than fifty cells, and the number of CD4 signals in the EGF ROIs was estimated.

Cell surface levels of CD4-furin and FLAG-TGN38 were estimated as follows. HeLa cells stably expressing CD4-furin or FLAG-TGN38 in a well of a 24-well plate were incubated with 15 mM sodium butyrate for 16 h to induce expression of the recombinant protein. The cells were then incubated with biotin-conjugated anti-CD4 or anti-FLAG antibody, respectively, at 4°C for 60 min, washed three times with PBS, and lysed in biotin cell lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100). After centrifugation of the lysate at 13,000 rpm in a microcentrifuge, the biotin-conjugated antibody in the supernatant was recovered with immobilized streptavidin beads (Pierce, Rockford, IL) according to the manufacturer's instructions. The beads were washed with biotin wash buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% Triton X-100) and with TBS-T (Tris-buffered saline containing 0.1% Tween 20), and incubated with HRP-conjugated anti-mouse IgG at room temperature for 30 min. The beads were then washed four times with TBS-T and developed with HRP substrate reagents (R&D Systems, Minneapolis, MN).

RESULTS

Simultaneous Knockdown of BIG2 and BIG1 Causes Redistribution of Transferrin Receptor and a Population of TGN-associated Proteins

In our previous study, we have shown that BIG1 is localized predominantly to the TGN and BIG2 is localized not only to

the TGN but also to recycling endosomes (Shin *et al.*, 2004); when HA-tagged BIG1 or BIG2 was expressed in HeLa cells, HA-BIG2 but not HA-BIG1 was significantly localized on peripheral punctate structures containing TfnR (a marker for early and recycling endosomes) and lacking EEA1 (an early endosomal marker; see Supplementary Figure S1). To explore roles specific for BIG2 or BIG1 and their redundant roles, we depleted HeLa cells of BIG2 or BIG1 alone or both ARF-GEFs by RNAi; as established in our previous study (Ishizaki *et al.*, 2006), BIG1, BIG2, or both proteins were almost completely depleted by transfecting pools of siRNAs into HeLa cells, as confirmed by immunoblotting (Supplementary Figure S2A) and immunofluorescence analysis (Figure S2B).

Knocking down BIG2 alone resulted in tubular extensions from peripheral punctate structures positive for TfnR and the AP-1 γ subunit (Figure 1, c and h), but did not affect perinuclear TGN-like structures for AP-1 (Figure 1h). The tubulation of the TfnR-containing compartment we observed in BIG2 knockdown cells is consistent with the tubulation observed in our previous study when dominant negative BIG2 was expressed and with that of Shen *et al.* by RNAi-mediated knockdown of BIG2 (Shin *et al.*, 2004; Shen *et al.*, 2006). BIG2 knockdown did not significantly alter the TGN localization of CD4-furin (a chimeric protein with the exoplasmic domain of CD4 and the transmembrane and cytoplasmic domains of furin; see Figure 6A), TGN46, golgin-245, or GGA3 (Figure 1, m and r, and Supplementary Figure S3).

We observed a similar redistribution of TfnR and AP-1, suggesting that BIG2 knockdown affects recycling endosomes. To confirm this, we examined the effects of BIG2 knockdown on the localization of endosomal Rab GTPases. As shown in Figure 2, knocking down BIG2 induced tubulation of structures containing Rab11 (c) but not those containing Rab4 (h) or Rab5 (m). Because Rab5, Rab4, and Rab11 are mainly associated with early, early/recycling, and recycling endosomes, respectively, and within the same recycling endosomes, Rab4 and Rab11 are associated with distinct subdomains (Sönnichsen *et al.*, 2000; Zerial and McBride, 2001), we concluded that BIG2 knockdown selectively induces tubulation of the Rab11-positive subdomains of recycling endosomes. This conclusion is apparently incompatible with our previous observations that overexpression of dominant-negative BIG2, BIG2(E738K), caused tubulation of Rab4-positive as well as Rab11-positive structures (Shin *et al.*, 2004). Although the exact reason for the differential effects of the BIG2 knockdown and the dominant-negative BIG2 overexpression is not clear, it is possible that the effects of BIG2(E738K) overexpression on the endosomal integrity is much more drastic than that of BIG2 depletion and thereby the BIG2(E738K) overexpression impairs the entire recycling endosomes, whereas the BIG2 depletion impairs only the Rab11-positive subdomains. In support of this possibility, the endosomal tubules induced by the BIG2(E738K) overexpression (Shin *et al.*, 2004) were much more prominent than those induced by the BIG2 depletion (Figure 2c). The more drastic effects of the BIG2(E738K) overexpression compared with the BIG2 depletion might result from sequestering not only ARFs through the mutated Sec7 catalytic domain but also unknown binding partners through regions outside of the Sec7 domain on the membrane.

Knocking down BIG1 alone did not significantly affect the localization of any of examined proteins (Figures 1, b, g, l and q, and 2, b, g and l, and Supplementary Figure S3).

Figure 1. Effects of knockdown of BIG2 and/or BIG1, or AP-1 on the localization of TGN and recycling endosomal proteins. HeLa cells (a–j and p–t) or HeLa cells stably expressing CD4-furin chimera (k–o; see *Materials and Methods*) were mock-treated (siRNAs for LacZ; a, f, k, and p) or treated with a pool of siRNAs for BIG1 (b, g, l, and q), BIG2 (c, h, m, and r), BIG1+BIG2 (d, i, n and s), or the AP-1 μ 1A subunit (e, j, o, and t), as described in *Materials and Methods*. The cells were then stained with antibody against TfnR (a–e), the AP-1 γ subunit (f–j), CD4 (k–o), or TGN46 (p–t). In panel s, asterisks indicate cells with reduced staining intensity for TGN46 and a plus sign indicates a cell with unaffected TGN46 staining.

Simultaneous knockdown of BIG2 and BIG1, however, resulted in considerable redistribution of not only recycling endosomal proteins but also a subset of TGN proteins; hereafter, we refer to simultaneous knockdown of BIG2 and BIG1 as “double knockdown.” As in cells lacking BIG2 alone, we observed tubulation of recycling compartments positive for TfnR, AP-1, and Rab11 in the double knockdown cells (Figures 1, d and i, and 2d). Furthermore, in the double knockdown cells, AP-1 was no longer found to be associated with the TGN (Figure 1i), suggesting that AP-1 recruitment to the TGN is due to ARF activation by BIG2 and BIG1. The staining for TGN46 disappeared in ~30% of the double knockdown cells (Figure 1s, cells indicated by

asterisks). Although we do not know the exact reason for the uneven redistribution of TGN46, we suspect this redistribution may require complete depletion of BIG2 and BIG1. Knocking down both BIG2 and BIG1 also alters the localization of CD4-furin. Specifically, its staining decayed and became more peripheral than that in control cells and often showed tubular appearance (Figure 1n). The disappearance of the TGN staining for AP-1 and TGN46 appeared to be specific effects of the double knockdown on these proteins but did not result from gross changes in Golgi morphology, because localization of other TGN markers (GGA3 and golgin-245) as well as *cis*-Golgi markers (GM130 and β -COP) was unaffected (Supplementary Figure S3).

Figure 2. Effects of knockdown of BIG2 and/or BIG1, or AP-1 on the localization of endosomal Rab proteins. HeLa cells transiently transfected with EGFP-Rab11 (a–e), EYFP-Rab4 (f–j) or EGFP-Rab5 (k–o) were mock-treated (a, f, and k) or treated with a pool of siRNAs for BIG1 (b, g and l), BIG2 (c, h and m), BIG1+BIG2 (d, i, and n), or the AP-1 μ 1A subunit (e, j, and o), as described in *Materials and Methods*.

Cell Surface Accumulation of CD4-Furin in Cells Depleted of Both BIG2 and BIG1

We next examined whether the morphological changes of recycling endosomes in cells depleted of BIG2 alone or in combination with BIG1 affect trafficking through these compartments. To this end, control or BIG-knockdown cells were incubated with AlexaFluor488-conjugated Tfn at 37°C for 60 min to allow its accumulation at early/recycling endosomes. After stripping surface-bound Tfn, the cells were then incubated at 37°C for appropriate time periods to follow its recycling through early/recycling endosomes. As shown in Supplementary Figure S4, we did not detect any significant difference in the recycling of fluorescent Tfn between the control and any of the BIG-knockdown cells. This is in line with previous observations that BFA, a specific inhibitor of ARF-GEFs of the Sec7/BIG and Gea/GBF groups (Jackson, 2000), causes gross tubulation of recycling endosomes but has marginal effects on Tfn recycling (Lippincott-Schwartz *et al.*, 1991). We also examined retrograde transport of Shiga toxin through recycling endosomes to the Golgi, but again did not observe any significant difference between the control and BIG-knockdown cells (data not shown). These results are comparable with our previous observations that expression of a dominant-negative mutant of BIG2, BIG2(E738K), did induce tubulation of recycling endosomes but did not significantly affect transport of Tfn or Shiga toxin through these compartments (Shin *et al.*, 2004). On the other hand, Shen *et al.* (2006) reported that release of accumulated Tfn from cells treated with BIG2 or BIG2+BIG1 siRNAs was slightly, but significantly, slower than from control cells. We do not know the reason for the difference between our data and that Shen *et al.*, because our protocols to determine the Tfn recycling are essentially the same as those of Shen *et al.* (2006). In any way, the effects of BIG2 depletion on the Tfn recycling may be marginal in spite of the considerable effects on the recycling endosome architecture. However, it is also possible that, in the BIG2-depleted cells, because of the impairment of these compartments, a predominant fraction of internalized Tfn is recycled to the cell surface by bypassing recycling endosomes (i.e., directly from early endosomes).

We then set out to explore the reason why the levels of CD4-furin and TGN46 in the TGN region were reduced in the double knockdown cells. We speculated that the reduced levels might result from mislocalization and/or degradation of these transmembrane proteins. We first compared steady-state levels of CD4-furin in the control and knockdown cells by immunoblot analysis of whole cell lysates but failed to detect any significant difference in the CD4-furin levels between the control and double knockdown cells (Supplementary Figure S5A). These results exclude the possibility that the furin construct was missorted to the degradation pathway in cells depleted of BIG2 and BIG1. We then estimated the level of CD4-furin that accumulated on the cell surface by incubating the cells with anti-CD4 antibody at 4°C before fixation. As shown in Figure 3A, the cell surface level of CD4-furin approximately doubled in the double knockdown cells compared with that in the control cells or those depleted of BIG1 or BIG2 alone.

In considerable contrast, there was no apparent difference in the cell surface level (Figure 3B) or total level (Supplementary Figure S5B) of N-terminally FLAG-tagged TGN38 between control and double knockdown cells when cells stably expressing this TGN38 construct were examined (TGN38 is a rat ortholog of human TGN46). However, when retrograde transport of FLAG-TGN38 from the cell surface

Figure 3. Cell surface levels of CD4-furin and FLAG-TGN38 in cells knocked down of BIG2 and/or BIG1, or AP-1. HeLa cells stably expressing CD4-furin (A) or FLAG-TGN38 (B) were mock-treated or treated with a pool of siRNAs for BIG1, BIG2, BIG1+BIG2, or μ 1A. The cell surface levels of CD4-furin and FLAG-TGN38 were estimated as described in *Materials and Methods*. (A) Data represent mean $\pm$ SD of three independent experiments. (B) Data from one experiment. ** $p < 0.01$. (C) FLAG-TGN38-expressing HeLa cells were mock-treated (left) or treated with a pool of siRNAs for BIG1+BIG2 (right), incubated with anti-FLAG M2 antibody at 19°C for 60 min to allow the antibody to accumulate in early endosomes, and chased for 90 min at 37°C. The cells were then stained for FLAG and golgin-97.

to the TGN was examined by monitoring extracellularly applied anti-FLAG antibody, a difference was observed between control and double knockdown cells (Figure 3C). Namely, in the control cells the anti-FLAG antibody, which was accumulated in early endosomes in advance, was predominantly transported to the TGN by a 90-min chase at 37°C (left), while the majority was retained in endosomal structures in the double knockdown cells (right). It is there-

Figure 4. Block in retrograde transport of CD4-furin at late endosomes by depletion of BIG2 and BIG1. HeLa cells stably expressing CD4-furin were mock-treated or treated with a pool of siRNAs for BIG1, BIG2, or BIG1+BIG2 as indicated. Subsequently, the cells were incubated with anti-CD4 antibody (Leu3a) alone (A and D) or a combination of the antibody and AlexaFluor488-conjugated EGF (B) at 19°C for 60 min to allow the antibody and fluorescent EGF to accumulate in early endosomes (top panels), then chased for 60 min at 37°C (bottom panels). The cells were incubated with anti-EEA1 (A) or anti-LBPA (D) antibody and then with secondary antibodies. In C, colocalization between the internalized CD4-furin and the EGF in the bottom panels in B was estimated as described in *Materials and Methods*. * $p < 0.05$, ** $p < 0.01$. In D, the Golgi signal of CD4-furin markedly decreased due to optimizing the immunofluorescence staining for LBPA.

fore likely that the disappearance of TGN46 in the TGN region in the double knockdown cells was due, at least in part, to a block in its retrograde transport to the TGN. The endosomal structures where the antibody was arrested were largely overlapped with EEA1 and TfnR and partially overlapped with Lamp-1 (data not shown).

Block in Retrograde Transport of CD4-Furin at Late Endosomes and Its Misrouting in Cells Depleted of Both BIG2 and BIG1

The high level of CD4-furin cell surface expression suggests a decrease in endocytosis or an increase in recycling of CD4-furin by knockdown of BIG2 and/or BIG1. To address these possibilities, we performed antibody uptake experiments in which retrograde transport of CD4-furin was monitored by following extracellularly applied anti-CD4 anti-

body. Internalization of CD4-furin from the cell surface to EEA1-positive early endosomes (antibody uptake at 19°C for 60 min) was not affected by the knockdown of either BIG2 or BIG1 or both (Figure 4A, top panels). However, further retrograde transport from early endosomes to the TGN was considerably delayed in cells depleted of both BIG2 and BIG1. In the control cells and those depleted of BIG2 or BIG1 alone, CD4-furin that accumulated in early endosomes was transported almost completely to perinuclear Golgi-like structures after temperature shift to 37°C for 60 min (Figure 4A, e–g, green). In marked contrast, a considerable fraction of CD4-furin remained in punctate endosome-like structures even after the temperature shift to 37°C in the double knockdown cells (Figure 4Ah; also see Figure 7Bc). However, the punctate CD4-furin labeling (green) was not significantly superimposed on the EEA1 staining (red), suggesting that

the CD4-furin molecules that transit through early endosomes accumulate in distinct endosomal compartments.

To identify the endosomal compartments where CD4-furin accumulates in the double knockdown cells, we compared the retrograde transport of CD4-furin with that of the epidermal growth factor (EGF) receptor, because a similar chimeric furin construct (Tac-furin) has been shown to be delivered to the TGN through late endosomes (Mallet and Maxfield, 1999; Schapiro *et al.*, 2004; see Figure 8A) and the EGF receptor is known to be transported through late endosomes to lysosomes for degradation in a ligand-dependent manner. When CD4-furin-expressing HeLa cells were incubated with anti-CD4 and fluorescently labeled EGF at 19°C for 60 min, both anti-CD4 (red) and EGF (green) accumulated on punctate early endosomal structures in the control and knockdown cells (Figure 4B, a–d). After temperature shift to 37°C for 60 min, the majority of anti-CD4 reached perinuclear Golgi region in control cells and those depleted of BIG2 alone (Figure 4B, e–g, red). In these cells, a considerable fraction of the EGF labeling disappeared because of its degradation in lysosomes, although a certain fraction was still found on punctate late endosomal structures (e–g, green). On the other hand, in the double knockdown cells (Figure 4Bh), the punctate structures containing CD4-furin (red) and late endosomes labeled by fluorescent EGF (green) showed a good codistribution. Note that transport of EGF through late endosomes to lysosomes for degradation was not apparently influenced by the double knockdown (Supplementary Figure S6). Quantitative estimation revealed that, after temperature shift to 37°C for 60 min, the amount of CD4-furin present within the EGF-positive endosomes in the double knockdown cells was approximately three times as much as that in the control cells; ~65% of internalized CD4-furin colocalized with internalized EGF in the double knockdown cells, whereas only ~20% colocalized in the control cells (Figure 4C). The quantitative estimation also revealed that, even in cells depleted of BIG1 alone and possibly those depleted of BIG2 alone, internalized CD4-furin tends to be trapped in EGF-positive endosomes to a

lesser extent (Figure 4C), although the single knockdown appeared not to significantly affect the steady-state localization of CD4-furin (Figure 1, l and m). These observations suggest that BIG1 and BIG2 are redundantly involved the retrograde transport from endosomes to the TGN, and BIG1 may contribute preferentially to this transport pathway.

To further define the late endosomal compartment where retrograde transport of internalized CD4-furin was blocked, we stained the CD4-furin-internalized cells with antibodies to some late endosomal markers. Although the punctate staining for CD4-furin accumulated in the double knockdown cells did not overlap with the staining for Lamp-1 (a marker for late endosomes and lysosomes) or Hrs (a marker for early and late endosomes; data not shown), it partially but significantly overlapped with the staining for LBPA (Kobayashi *et al.*, 1998, 1999), a marker for late endosomes/multivesicular bodies (Figure 4D). These observations indicate that knocking down both BIG2 and BIG1 blocks retrograde transport to the TGN of the furin construct at some population of late endosomes.

Taking into account the data that the cell surface level of CD4-furin is elevated (Figure 3A) whereas its total level is unchanged (Supplementary Figure S5A) in the double knockdown cells compared with control cells, it is likely that the block in retrograde transport of CD4-furin resulted in its recycling to the cell surface (see Figure 8B). In support of this speculation, a population of CD4-furin was found along TfnR-positive tubules derived from recycling endosomes in the double knockdown cells (Figure 5).

Defects in Retrograde Transport of CD4-Furin in the Cells Depleted of BIG2 and BIG1 Resemble Those Depleted of AP-1

During the course of these experiments, we noticed that the block in retrograde transport of CD4-furin in the double knockdown cells resembles the block in retrograde transport of furin constructs with mutation(s) in its cytoplasmic domain (Jones *et al.*, 1995; Schäfer *et al.*, 1995; Takahashi *et al.*, 1995; Voorhees *et al.*, 1995; reviewed in Thomas, 2002). In

Figure 5. Presence of TfnR and CD4-furin on the same tubular structures induced by depletion of BIG2 and BIG1. HeLa cells stably expressing CD4-furin were mock-treated (a–a'') or treated with pools of siRNAs for BIG1 and BIG2 (b–b'') and stained for TfnR (a and b) and CD4 (a' and b'). Merged images are shown in a'' and b''. Enlarged images of b–b'' are shown in the bottom panels.

Figure 6. Retrograde transport of CD4-furin with mutations within the furin cytoplasmic domain. (A) Schematic representation of the structure of CD4-furin and the primary sequence of the furin cytoplasmic domain. Substituted residues within the furin cytoplasmic domain in the mutants, YA, SA, and YA/SA, are indicated. (B) HeLa cells transiently transfected with the indicated CD4-furin construct were incubated with anti-CD4 antibody and AlexaFluor488-conjugated EGF and processed for detection of the CD4 antibody as described in the legend for Figure 4B.

previous studies, we and others have shown that steady-state localization to the TGN and recycling from the cell surface of furin both involve a Tyr-based motif, YKGL, and an acidic cluster sequence, SDSEEDE, containing Ser residues that are phosphorylated by protein kinase CK2, within its cytoplasmic domain (Figure 6A). As shown in Figure 6B, a CD4-furin construct with a WT cytoplasmic domain underwent retrograde transport to the TGN (a), whereas transport of constructs with mutations at both the Tyr and Ser residues within the cytoplasmic domain, YA/SA, were blocked at late endosomes (b–d).

The steady-state localization and retrograde trafficking to the TGN of furin has been proposed to involve the association of the AP-1 complex with the acidic cluster and the Tyr-based motif (Dittie *et al.*, 1997; Wan *et al.*, 1998; Teuchert *et al.*, 1999; Crump *et al.*, 2001). Moreover, AP-1 is recruited to membranes in an ARF-dependent manner (Stamnes and Rothman, 1993; Zhu *et al.*, 1998; Austin *et al.*, 2000). We therefore decided to examine the effects of AP-1 (μ 1A subunit) depletion on the localization and retrograde trafficking of CD4-furin as well as the localization of other proteins. Even though we could not confirm depletion of the μ 1A subunit due to unavailability of an anti- μ 1A antibody, the level of the γ subunit was specifically decreased in the μ 1A-knockdown cells (Figures 1j and 7A), probably due to its instability from the lack of the μ 1A subunit in the AP-1 complex. In the AP-1 knockdown cells, as in cells depleted of both BIG2 and BIG1, CD4-furin was distributed more peripherally than in control cells and was found on punctate, often tubular, endosome-like structures (Figure 10), and its abundance at the cell surface was increased (Figure 3A). These observations are in line with those of the previous studies obtained using cells derived from AP-1 knockout mice and using AP-1 knockdown cells (Meyer *et al.*, 2001; Hirst *et al.*, 2005). Furthermore, TfnR and Rab11 redistributed into tubular structures in the AP-1 knockdown cells (Figures 1e and 2e) as in the double knockdown cells. However, the steady-state localization of other Golgi and TGN proteins examined was unaffected in the AP-1-depleted cells as in cells depleted of BIG2 and BIG1 (Supplementary Figure S3).

We then examined if the AP-1 depletion affects the retrograde trafficking of CD4-furin. As shown in Figure 7, B and C, in the AP-1-depleted cells a fraction of the internalized anti-CD4 antibody was arrested at punctate late endosomal compartments positive for endocytosed EGF (Figure 7B, b and b'), as in the cells depleted of BIG2 and BIG1 (Figure 7B, c and c').

DISCUSSION

BIG1 and BIG2 are closely related ARF-GEFs that were originally copurified as part of a large complex and were coimmunoprecipitate with each other, suggesting that they exist in the same protein complex (Morinaga *et al.*, 1996; Yamaji *et al.*, 2000). Cherfils and colleagues recently showed that N-terminal DCB and HUS domains of BIG1, BIG2, and GBF1 are important for intra- and intermolecular interactions (Mouratou *et al.*, 2005; Ramaen *et al.*, 2007), suggesting that BIG1 and BIG2 are capable of functioning as homomeric or heteromeric complexes. On the other hand, we previously revealed that BIG2 but not BIG1 is required for the integrity of recycling endosomes. These data led us to examine how the specific or redundant functions of BIG1 and BIG2 are regulated in a cellular context by examining relevant subcellular compartments after RNAi-mediated knockdown of these proteins. We failed to detect any obvious effect of BIG1 knockdown on the localization of TGN and endosomal proteins and on trafficking through these compartments. In contrast, BIG2 knockdown induces tubular extensions from recycling endosomes positive for TfnR, AP-1, and Rab11. Knocking down both BIG2 and BIG1 abolishes TGN-localization of AP-1 and TGN46 and causes redistribution, often into tubular endosomal structures, of CD4-furin and AP-1, in addition to inducing the phenotype observed in the cells knocked down of BIG2 alone. Moreover, we made essentially the same observations in AP-1 knockdown cells as for those (but for AP-1 localization) in the double knockdown cells.

Our data allow us to formulate a model that: 1) BIG2 functions at recycling endosomes, and BIG2 and BIG1 play redundant roles at the TGN; and 2) these ARF-GEFs activate ARFs at these compartments, and the activated ARFs in turn regulate AP-1 functions.

The simultaneous depletion of BIG2 and BIG1 appears not to affect anterograde transport of vesicular stomatitis virus G protein (VSVG) from the TGN to the plasma membrane (data not shown). We and Togawa *et al.* (1999) previously showed that BIG1 and BIG2 have a GEF activity toward ARF1 and ARF3 (see also Shin *et al.*, 2004), and Volpicelli-Daley *et al.* (2005) observed that simultaneous knockdown of ARF1 and ARF3 shows marginal effects on VSVG transport to the plasma membrane. These data support our observation that the double knockdown of BIG2 and BIG1 does not affect the anterograde transport of VSVG from the TGN. It is thus unlikely that ARF activation by BIG2 and BIG1 at the

Figure 7. BIG2+BIG1 and AP-1 knockdown cells show a similar phenotype in terms of retrograde transport of CD4-furin. (A) HeLa cells mock-treated or treated with a pool of siRNAs for the μ 1A subunit of the AP-1 complex were subjected to immunoblot analysis for detection of the γ -adaptin subunit of the AP-1 complex and the μ 3A subunit of the AP-3 complex as a control. (B) HeLa cells stably expressing CD4-furin were mock-treated (a and a') or treated with a pool of siRNAs for μ 1A (b and b') or BIG1+BIG2 (c and c'). Subsequently, the cells were incubated with anti-CD4 antibody alone (a–c) or a combination of the antibody and AlexaFluor488-conjugated EGF (a'–c') at 19°C for 60 min, then chased for 60 min at 37°C and processed for detection of the CD4 antibody. (C) Colocalization of the internalized CD4-furin and EGF in B (bottom panels), was quantitatively estimated as described under the legend for Figure 4C. ** $p < 0.01$.

TGN is required for VSVG transport to the plasma membrane.

Volpicelli-Daley *et al.* (2005) also reported that simultaneous knockdown of ARF1 and ARF3 significantly retarded Tfn recycling. Our preliminary analysis using the same shRNA constructs as those used by Volpicelli-Daley *et al.* also revealed a tendency that internalized Tfn is accumulated in cells depleted of ARF1 and ARF3. Given that double knockdown of BIG1 and BIG2 does not significantly affect Tfn recycling (Figure S4) albeit leading to marked morphological changes of recycling endosomes (Figures 1, d and i, and 2d), whereas simultaneous knockdown of ARF1 and ARF3 causes both the recycling block and the morphological changes, it is possible that the BIG1+BIG2 knockdown affects recycling through recycling endosomes alone, whereas the ARF1+ARF3 knockdown affects recycling through not only recycling endosomes but also early endosomes. Taken together with a previous report showing that BFA has marginal effects on Tfn recycling albeit causing endosomal tubulation (Lippincott-Schwartz *et al.*, 1991), a BFA-insensitive ARF-GEF(s) might be responsible for activation of ARFs that participate in recycling of Tfn through early endosomes.

The double knockdown of BIG2 and BIG1 results in redistribution of a subset of TGN-localizing proteins (Figure 1 and Supplementary Figure S3). To our surprise, however,

the double knockdown does not affect the localization of GGA3, which associates with the TGN in an ARF-dependent manner (for reviews, see Nakayama and Wakatsuki, 2003; Bonifacio, 2004). A most recent report could provide an explanation for this observation: Lefrançois and McCormick (2007) showed that GBF1 interacts with GGAs and is required for their recruitment onto Golgi membranes. Therefore, ARFs activated by BIG2 and BIG1 at the TGN recruits specific coat proteins and regulates transport of specific cargo proteins.

The simultaneous knockdown of BIG2 and BIG1 does not apparently affect retrograde transport of Shiga toxin or recycling of Tfn via recycling endosomes, despite considerable morphological changes in these compartments. Intriguingly, the double knockdown blocks retrograde transport of CD4-furin to the TGN, and consequently increases its accumulation in peripheral endosomes (Figure 4) and its expression on the cell surface (Figure 3A). It is therefore likely that, through activating ARFs, BIG2 and BIG1 play a crucial role in retrograde transport from endosomes to the TGN. The block in retrograde transport of CD4-furin at late endosomes appears to cause its recycling to the cell surface rather than its missorting to the lysosomal degradation pathway (Figure 8B) for the following reasons. First, we do not observe colocalization of internalized CD4-furin and lysosomal markers,

Figure 8. Models of retrograde transport pathways of furin in the control cells (A) and in cells knocked down of both BIG1 and BIG2 (B).

such as Lamp-1, at any time point (data not shown). Second, in the double knockdown cells, the total amount of CD4-furin is not significantly changed (Supplementary Figure S5), whereas its cell surface expression is increased (Figure 3A). Finally, the recycling pathway appears not to be affected in the double knockdown cells (Supplementary Figure S4).

Robinson and colleagues showed that knocking down AP-1 or its accessory proteins causes redistribution of another furin chimera, CD8-furin, to cell peripheral structures and the plasma membrane (Hirst *et al.*, 2004, 2005). Using a Tac-furin construct, Maxfield and colleagues carefully examined the role of the cytoplasmic domain of furin in its trafficking from the plasma membrane to the TGN through endosomal compartments. Their data suggested that phosphorylation of Ser residues in the acidic cluster sequence within the cytoplasmic domain plays an important role in selective sorting of Tac-furin into late endosomes and in retrieval from late endosomes to the TGN (Mallet and Maxfield, 1999; Schapiro *et al.*, 2004). The behavior of CD4-furin we observe in the double knockdown cells and in AP-1 knockdown cells closely resembles that of cytoplasmic domain mutants with substitutions of the phosphorylatable Ser residues to unphosphorylatable Ala. Taken together with our data, it is likely that ARFs activated by BIG2 and BIG1 regulate AP-1, after which AP-1 regulates retrograde transport of furin.

Our attempts to unequivocally define the compartments accumulating CD4-furin in cells depleted of both BIG2 and BIG1 or those depleted of AP-1 have not been successful. Given that CD4-furin colocalizes with LBPA and internalized EGF but not with EEA1, Lamp-1, or internalized Tfn (Figure 4 and our unpublished observations), the CD4-furin-accumulating compartments are very likely to be late endosomes and/or intermediates of early and late endosomes. It is possible that BIG2 and BIG1 play a redundant roles at late endosomal compartments, although we have so far failed to show localization of BIG2 or BIG1 at these compartments because of limitations in the sensitivity of our anti-BIG2 and anti-BIG1 antibodies (Ishizaki *et al.*, 2006).

Similar to CD4-furin, the staining for TGN46 is reduced in the double knockdown cells (Figure 1). A simple explanation for this observation is that retrograde transport of TGN46 from endosomes to the TGN is inhibited by the depletion of

BIG2 and BIG1. As expected, we found that in the double knockdown cells retrograde transport of FLAG-TGN38 (TGN38 is a rat ortholog of human TGN46) to the TGN is inhibited (Figure 3C), although compartments accumulating internalized FLAG-TGN38 are not significantly overlapped with those positive for internalized EGF (data not shown), and the cell surface level of FLAG-TGN38 was not significantly changed (Figure 3B). The behavioral difference between CD4-furin and FLAG-TGN38 may be explained by the different retrograde routes taken by them, as previously shown by Mallet and Maxfield (1999). One of candidate regulators that discriminate between the retrograde transport of furin and TGN38 is PACS-1 (phosphofurin acidic cluster sorting protein-1). Thomas and colleagues proposed that PACS-1 is a critical connector between AP-1 and the acidic cluster containing phospho-Ser residues within the furin cytoplasmic domain and regulates steady-state distribution and trafficking of furin (Wan *et al.*, 1998; Crump *et al.*, 2001; Thomas, 2002). A recent RNAi study of Robinson and colleagues, however, has indicated that, for the furin localization and trafficking, PACS-1 is dispensable even though the acidic cluster is indeed the essential sorting signal and AP-1 and clathrin do play critical roles (Lubben *et al.*, 2007). Our attempts to show a role of PACS-1 in trafficking of CD4-furin have been also unsuccessful so far (data not shown). Thus, the function of PACS-1 is currently unclear, and although not essential, it might play some sort of a regulatory role in the furin localization and trafficking.

In the present study, we demonstrate the first evidence that BIG2 and BIG1 play redundant roles in recruitment of AP-1 and in the retrograde transport from endosomes to the TGN. Moreover, we have determined the specific function of BIG2 for the integrity of recycling endosomes. Our study leads to further questions, namely, whether BIG1 and BIG2 carry out their redundant and nonredundant functions in a spatially and temporally regulated manner and how the formation of homomeric and heteromeric complexes is linked to the regulation of the function and localization of BIG1 and BIG2.

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