BIG2, A Guanine Nucleotide Exchange Factor for ADP-Ribosylation Factors: Its Localization to Recycling Endosomes and Implication in the Endosome Integrity[D]

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Small GTPases of the ADP-ribosylation factor (ARF) family play a key role in membrane trafficking by regulating coated vesicle formation, and guanine nucleotide exchange is essential for the ARF function. Brefeldin A blocks the ARF-triggered coat assembly by inhibiting the guanine nucleotide exchange on ARFs and causes disintegration of the Golgi complex and tubulation of endosomal membranes. BIG2 is one of brefeldin A-inhibited guanine nucleotide exchange factors for the ARF GTPases and is associated mainly with the *trans*-Golgi network. In the present study, we have revealed that another population of BIG2 is associated with the recycling endosome and found that expression of a catalytically inactive BIG2 mutant, E738K, selectively induces membrane tubules from this compartment. We also have shown that BIG2 has an exchange activity toward class I ARFs (ARF1 and ARF3) in vivo and inactivation of either ARF exaggerates the BIG2(E738K)-induced tubulation of endosomal membranes. These observations together indicate that BIG2 is implicated in the structural integrity of the recycling endosome through activating class I ARFs.

INTRODUCTION

Membrane traffic between organelles is mediated by vesicular and tubular intermediates that selectively carry cargo proteins and lipids from one compartment to another under the control of a variety of proteins. Small GTPases of the ADP-ribosylation factor (ARF) family trigger budding of coated vesicles by recruiting coat protein complexes onto organelle membranes, such as COPI onto the *cis*-Golgi, AP-1/clathrin onto the *trans*-Golgi network (TGN) and endosomes, AP-3 onto endosomes, and Golgi-localizing, γ-adaptin ear homology domain, ARF-binding proteins (GGAs) onto the TGN (Stamnes and Rothman, 1993; Traub et al., 1993; West et al., 1997; Lippincott-Schwartz et al., 1998; Ooi et al., 1998; Spang et al., 1998; Dell'Angelica et al., 2000). Similar to other GTPases, ARF switches between a GDP-bound (inactive) state and a GTP-bound (active) state, in which it

Abbreviations used: ARF, ADP-ribosylation factor; BFA, brefeldin A; CI-MPR, cation independent-mannose-6-phosphate receptor; EE, early endosome; EEA1, early endosomal autoantigen; GEF, guanine nucleotide exchange factor; GGA, Golgi-localizing, γ-adaptin ear homology domain, ARF-binding protein; Lamp-1, lysosome-associated membrane protein-1; LE, late endosome; RE, recycling endosome; Stx, Shiga toxin; Tfn, transferrin; TGN, *trans*-Golgi network.

transmits signals to its effectors. The exchange of GDP for GTP is catalyzed by guanine nucleotide exchange factors (GEFs). ARF-GEFs identified so far can be divided into four subfamilies: the Gea/GBF/GNOM, Sec7/BIG, ARNO/cytohesin/GRP, and EFA6 subfamilies. The Gea/GBF/GNOM subfamily includes yeast Gea1p and Gea2p, mammalian GBF1, and plant GNOM, and the Sec7/BIG subfamily includes yeast Sec7p and mammalian BIG1 and BIG2. Members of these two subfamilies have been shown to regulate membrane traffic by activating ARFs. They all share a catalytic region of ~200 amino acids termed the Sec7 domain (Peyroche et al., 1996), which is the molecular target of brefeldin A (BFA) (Chardin et al., 1996; Peyroche et al., 1999; Jackson and Casanova, 2000). Among these ARF-GEFs, members of the Sec7/BIG subfamily, and Gea1p, Gea2p, and GNOM are sensitive to BFA (Peyroche et al., 1999; Geldner et al., 2003). BFA causes apparent disintegration of the Golgi complex and tubulation of endosomal membranes, at least in part, by inhibiting the activation of the ARF family by the GEFs and in consequence inhibiting coated vesicle budding (Donaldson et al., 1990; Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Tooze and Hollinshead, 1991; Wood et al., 1991; Robinson and Kreis, 1992; Wood and Brown, 1992).

The endosomal compartments receive both endocytic cargoes from the plasma membrane and biosynthetic cargoes from the TGN. The existence of two routes connecting the endocytic and biosynthetic pathways have been suggested (Rohn et al., 2000). The first route links the late endosome (LE) and the TGN. A series of studies revealed the molecular mechanisms underlying retrieval of cation-independent mannose 6-phosphate receptor (CI-MPR) from the LE to the TGN via this route, which is regulated by the small GTPase Rab9 and its effector TIP47 and facilitated by α-SNAP, a component of the SNARE machinery (Itin et al., 1997; Carroll

et al., 2001). Morphological and biochemical studies indicated the existence of a second route, by which TGN38/TGN46, a cellular protein known to cycle between the TGN and the plasma membrane, and the Shiga toxin (Stx) traffic directly from the early/recycling endosome (EE/RE) to the TGN by bypassing the late endocytic pathway (Mallet and Maxfield, 1999). This newly described route may involve Rab11 (Wilcke *et al.*, 2000), the SNARE complex, and a Rab6 isoform (Mallard *et al.*, 2002). However, the molecular mechanisms underlying the EE/RE-to-TGN transport are poorly understood.

Rab4, Rab5, and Rab11 are well-known regulators of the endocytic and recycling pathways (Bucci *et al.*, 1992; van der Sluijs *et al.*, 1992; Ullrich *et al.*, 1996; Ren *et al.*, 1998). These Rab GTPases are localized to certain endosomal compartments but restricted to distinct regions of the membrane that may define specialized functional membrane domains (Sönnichsen *et al.*, 2000; Zerial and McBride, 2001; De Renzis *et al.*, 2002; Miaczynska and Zerial, 2002). The domains also can be distinguished by their BFA sensitivity, namely, the Rab4/Rab11-positive, but not the Rab5/Rab4-positive, domains of endosomes are sensitive to this drug (Sönnichsen *et al.*, 2000), suggesting that the former domains involve a BFA-sensitive ARF-GEF.

We previously showed that BIG2 is responsible for recruitment of the AP-1 clathrin adaptor complex, but not that of the COPI complex, onto membranes (Shinotsuka *et al.*, 2002b). More importantly, we found that expression of a catalytically inactive mutant of BIG2 induces membrane tubulation like the BFA treatment. However, the membrane tubules induced by the BIG2 mutant did not contain *cis*/medial- or *trans*-Golgi markers (e.g., β-COP, mannosidase II, and TGN46) (Shinotsuka *et al.*, 2002a).

Here, we show that BIG2 localizes to the RE as well as to the TGN and the catalytically inactive BIG2 mutant selectively induces tubulation of the RE, but not that of the EE, LE, or the Golgi complex. Furthermore, we have found that BIG2 specifically activates class I ARFs (ARF1 and ARF3) in vivo and that inactivation of ARF1 or ARF3 exaggerates the membrane tubule formation induced by the BIG2 mutant.

MATERIALS AND METHODS

Plasmids

Construction of expression vectors for hemagglutinin (HA)-tagged and untagged BIG2 (Shinotsuka *et al.*, 2002b) was described previously. Expression vectors for enhanced green fluorescent protein (EGFP)-tagged Rab4 and Rab5, and those for enhanced cyan fluorescent protein (ECFP)-tagged Rab7 and Rab11 (Sönnichsen *et al.*, 2000) were kindly provided by Marino Zerial (Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany), and that for FLAG-tagged TGN38 was by Nobuhiro Nakamura (Kanazawa University, Ishikawa, Japan).

Reagents and Antibodies

Stx1 was purified from *Escherichia coli* MC1061, by using pigeon egg ovomucoid affinity column chromatography (Miyake *et al.*, 2002); *E. coli* Stx1 is identical to Stx from *Shigella dysenteriae* in the primary sequence. Stx1 was labeled with Cy3 by using a Flurolink-antibody Cy3-labeling kit (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom) as described in the manufacturer's instructions. Alexa488-conjugated epidermal growth factor (EGF) and transferrin (Tfn) were purchased from Molecular Probes (Eugene, OR). Anti-BIG2 antisera were raised in rabbits against a synthetic peptide RLKHSQAQSKPTTPEKTDL(C) (Yamaji *et al.*, 2000) conjugated with keyhole limpet hemocyanin. The sera were affinity-purified using the peptide immobilized on Sulfolink beads (Pierce Chemical, Rockford, IL). Polyclonal rabbit antibodies against TGN46 (Kain *et al.*, 1998) and Golgin-97 (Yoshimura *et al.*, 2004) were kindly provided by Minoru Fukuda (Burnham Institute, La Jolla, CA) and Nobuhiro Nakamura (Kanazawa University), respectively. Monoclonal mouse antibodies against Tfn receptor (TfnR) (H68.4) were purchased from Zymed Laboratories (South San Francisco, CA); FLAG (M2) and γ-adaptin (100.3) were from Sigma-Aldrich (St. Louis, MO);

c-Myc (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA); CI-MPR (2G11) was from Affinity Bioreagents (Golden, CO); and lysosome-associated membrane protein-1 (Lamp-1) (H4A3), GGA3 (clone 8), and EEA1 (clone 14) were from BD Biosciences (San Jose, CA). Monoclonal rat anti-HA antibody (3F10) was from Roche Diagnostics (Indianapolis, IN). Polyclonal rabbit antibodies against β-COP were from Affinity Bioreagents. Alexa488-conjugated secondary antibodies were purchased from Molecular Probes, and Cy3-, Cy5-and peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture, Transfection, and Immunofluorescence Analysis

HeLa cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells grown to ~30% confluence were transfected with appropriate plasmids by using a FuGENE6 reagent according to the manufacturer's instructions (Roche Diagnostics). After 20 h, the transfected cells were fixed with 3% paraformaldehyde and processed for immunofluorescence labeling as described previously (Shin *et al.*, 1997).

For EGF or Tfn internalization, HeLa cells expressing HA-tagged BIG2 were grown on glass coverslips and cultured for 3 h in serum-free medium supplemented with 0.2% bovine serum albumin to deplete endogenous EGF or Tfn. The cells were then incubated with Alexa488-conjugated EGF or Tfn for indicated times and fixed with 3% paraformaldehyde. The cells were stained with monoclonal rat anti-HA antibody and then Cy3-conjugated anti-rat secondary antibody. For Stx1 or antibody internalization, HeLa cells expressing HA-tagged BIG2 alone or in combination with FLAG-tagged TGN38 were grown on glass coverslips and incubated with Cy3-conjugated Stx1, or anti-FLAG antibody for 50 min on ice. The cells were washed with phosphate-buffered saline (PBS) and then incubated at 37°C for indicated times in normal medium. The cells were then fixed and stained with monoclonal rat anti-HA antibody followed by Alexa488-conjugated anti-rat secondary antibodies. In the case of antibody internalization experiments, the cells were stained with monoclonal rat anti-HA antibody followed by Cy3-conjugated anti-rat and Alexa488-conjugated anti-mouse secondary antibodies.

For Tfn recycling, HeLa cells expressing HA-BIG2 were serum starved for 3 h and incubated with Alexa488-conjugated Tfn for 60 min at 37°C. The cells were then extensively washed with PBS to remove unbound Tfn and were further incubated at 37°C in normal medium for indicated times. Immunofluorescence staining was performed as described above.

ARF Pull-Down Assay

To determine the GEF activity of BIG2, we made use of the ARF pull-down assay as described previously (Santy and Casanova, 2001; Shinotsuka *et al.*, 2002b). The GAT domain of GGA1 fused to the C terminus of glutathione *S*-transferase (GST) (Takatsu *et al.*, 2002) was expressed in *E. coli* BL21(DE3) cells and purified using glutathione-Sepharose 4B beads (Amersham Biosciences UK) as described in the manufacturer's instructions.

HeLa cells grown on a 10-cm plate were transfected with an expression vector for C-terminally HA-tagged ARF in combination with that for HA-tagged BIG2(WT) or an empty vector by using a FuGENE6 reagent and incubated for 20 h. The cells were then lysed in 0.65 ml of cell lysis buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM MgCl$_2$, 0.5% sodium deoxycholate, 1% Triton X-100, and 10% glycerol) containing a Complete-EDTA free, protease inhibitor mixture (Roche Diagnostics) and centrifuged at maximum speed in a microcentrifuge for 20 min at 4°C. The supernatant was precleared with glutathione-Sepharose 4B beads and then incubated with the GST-GGA1-GAT domain (~40 μg) prebound to glutathione-Sepharose 4B beads for 1 h at 4°C and centrifuged at 2000 × *g* for 5 min at 4°C. The pellet was washed three times with wash buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM MgCl$_2$, 1% NP-40, and 10% glycerol) containing Complete-EDTA free. The bound materials were eluted by boiling in an SDS-PAGE sample buffer and resolved on a 15% SDS-polyacrylamide gel for detection of ARF or 7% SDS-polyacrylamide gel for detection of BIG2 and subjected to Western blot analysis. Results were quantified using the Image Gauge software program (Fuji Photo Film, Tokyo, Japan).

RESULTS

BIG2 Localization to TfnR-positive Endosomes

We and others previously showed that BIG2 is localized mainly to the TGN but some population also is associated with punctate structures distributed throughout the cytoplasm (Yamaji *et al.*, 2000; Shinotsuka *et al.*, 2002b). To determine whether these peripheral punctate structures correspond to endosomes, wild-type BIG2 [BIG2(WT)] was expressed in HeLa cells, and its intracellular localization was analyzed by double staining with antibodies against several organelle-specific proteins (Figure 1). As expected, the pe-
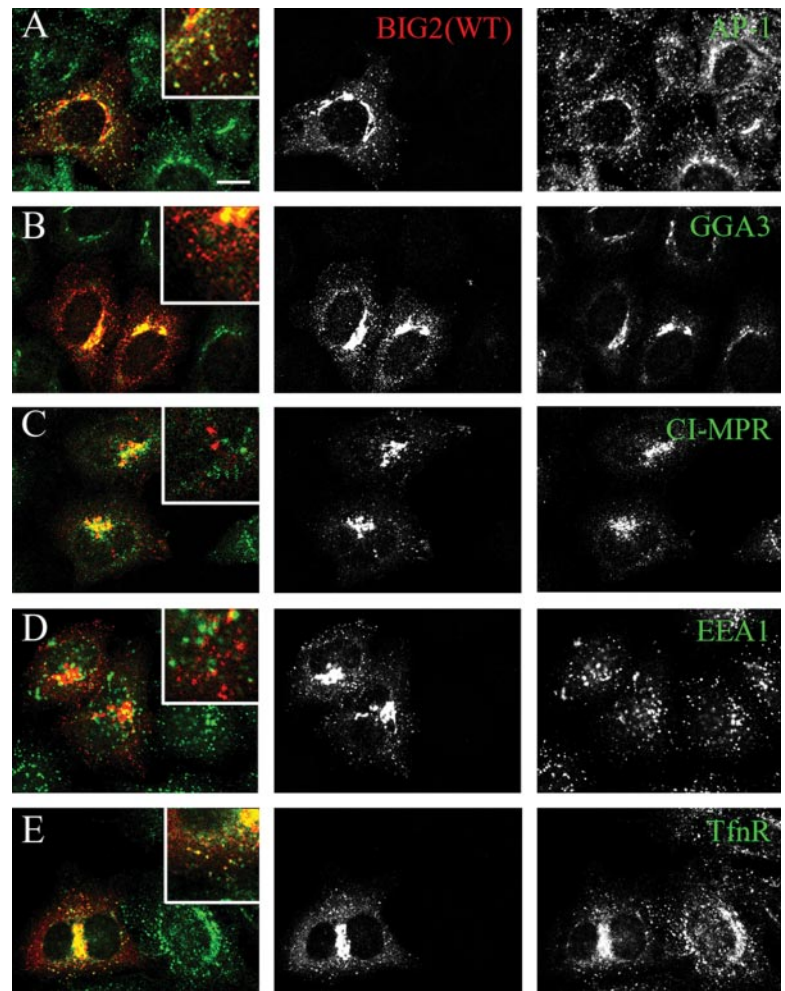
Figure 1. Localization of BIG2. HeLa cells expressing HA-tagged BIG2(WT) were fixed and double stained for HA and either the AP-1 γ-adaptin subunit (A), GGA3 (B), CI-MPR (C), EEA1 (D), or TfnR (E) followed by Cy3-conjugated anti-rat IgG and Alexa488-conjugated anti-mouse IgG.

rinuclear staining for BIG2 was superimposed on that for GGA3 (B), which is one of the clathrin adaptors, associated with the TGN and involved in the MPR transport, and on that for CI-MPR (C), which cycles between the TGN and the LE, indicating the TGN localization of BIG2. However, the peripheral punctate staining for BIG2 was not significantly overlapped with that for CI-MPR (Figure 1C, inset). Furthermore, the punctate BIG2 staining was overlapped neither with that for an early endosomal marker, EEA1 (Figure 1D, inset), nor with that for a late endosomal/lysosomal marker, Lamp-1 (our unpublished data). In striking contrast, BIG2 was extensively colocalized with AP-1 not only on the TGN but also on the punctate structures throughout the cytoplasm (Figure 1A, inset). In addition, the BIG2 staining was overlapped with that for TfnR (a marker for the EE and RE) on the peripheral punctate structures (Figure 1E, inset) as well as in the perinuclear region. These observations indicate that BIG2 is associated not only with the TGN (Shinotsuka *et al.*, 2002b) but also with the AP-1– and TfnR-positive endosomal compartments.

Tubulation of BIG2-positive Endosomes by Rab11(S25N) Expression

The TfnR-positive compartment was shown to be tubulated by overexpression of a dominant-negative Rab11 mutant, S25N (Wilcke *et al.*, 2000). We therefore reasoned that if BIG2 is associated with the TfnR-positive compartment, the BIG2-

positive structures also are tubulated by the Rab11(S25N) overexpression. To test this idea, HeLa cells cotransfected with BIG2(WT) and Rab11(S25N) were allowed to internalize Alexa488-conjugated Tfn for 60 min at 37°C to label the RE and immunostained for BIG2. As expected, BIG2 and internalized Tfn were colocalized on tubules emanating from peripheral punctate structures in the Rab11(S25N)-expressing cells (Figure 2A, inset), indicating that BIG2 is associated with the TfnR-positive recycling compartment as well as with the TGN.

Membrane Tubules Induced by Expression of a Catalytically Inactive BIG2 Mutant

The functional importance of the Sec7 domain was first suggested in *Arabidopsis thaliana*. Shevell *et al.* (1994) reported that mutations in the *EMB30* (also known as *GNOM*) gene locus caused defects in apical-basolateral pattern formation. More importantly, they found that one of the mutant alleles, *emb30-1*, has a mutation in the Sec7 domain that alters a conserved Glu residue to a Lys, E658K. Following x-ray crystallographic and mutational analyses revealed that the Glu residue conserved in the Sec7 domains of all ARF-GEFs plays a pivotal role in the guanine-nucleotide exchange reaction (Cherfils *et al.*, 1998; Mossessova *et al.*, 1998) and that a mutation of the Glu residue in ARNO, E156K, stabilizes the complex of the Sec7 domain with GDP-bound ARF without inducing GDP release (Renault *et al.*, 2003).
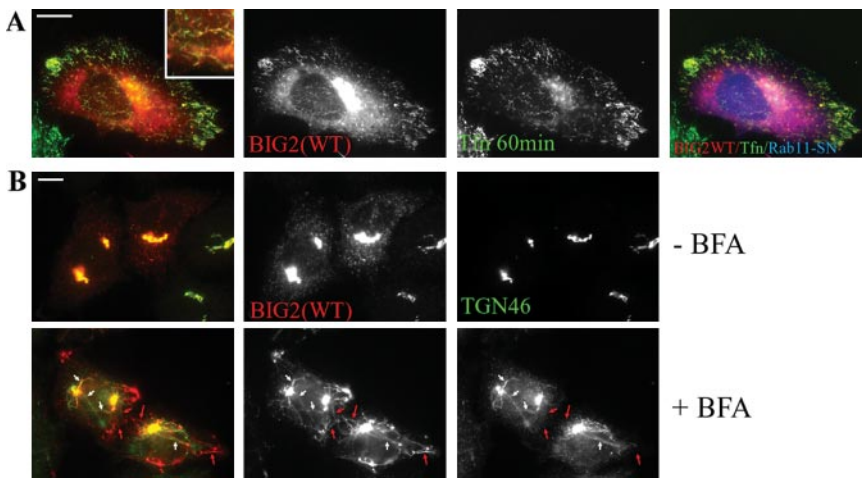
Figure 2. Effects of Rab11(S25N) expression and BFA treatment. (A) HeLa cells expressing HA-tagged BIG2(WT) and FLAG-tagged Rab11(S25N) were serum starved and allowed to internalize Alexa488-conjugated Tfn for 60 min at 37°C as described under *Materials and Methods*. The cells were fixed and stained with rat monoclonal anti-HA and mouse monoclonal anti-FLAG antibodies followed by Cy3-conjugated anti-rat and Cy5-conjugated anti-mouse secondary antibodies. (B) HeLa cells expressing HA-tagged BIG2(WT) were treated with vehicle only (top) or with 5 μg/ml BFA (bottom) for 15 min and then fixed and double stained for HA and TGN46 followed by Cy3-conjugated anti-rat and Alexa488-conjugated anti-rabbit secondary antibodies. Bar, 10 μm.

Therefore, BIG2(E738K), a mutant with a replacement of the corresponding Glu residue by Lys, is expected to be catalytically inactive. We previously showed that, like BFA treatment of cells, overexpression of BIG2(E738K) induces membrane tubules (Shinotsuka *et al.*, 2002a). We therefore examined which subcellular compartment was tubulated by the BIG2(E738K) expression. As shown in Figure 3, BIG2(E738K)-induced tubular structures were found mainly in the cell periphery. In contrast, the perinuclear structures positive for the BIG2(E738K) seemed not to have significant morphological difference from those for BIG2(WT) (compare Figures 1 and 3). In cells with moderate expression levels of BIG2(E738K), TGN46 (Figure 3C), Golgin-97 (Supplementary Figure S1B) and GGA3 (Supplementary Figure S1C)
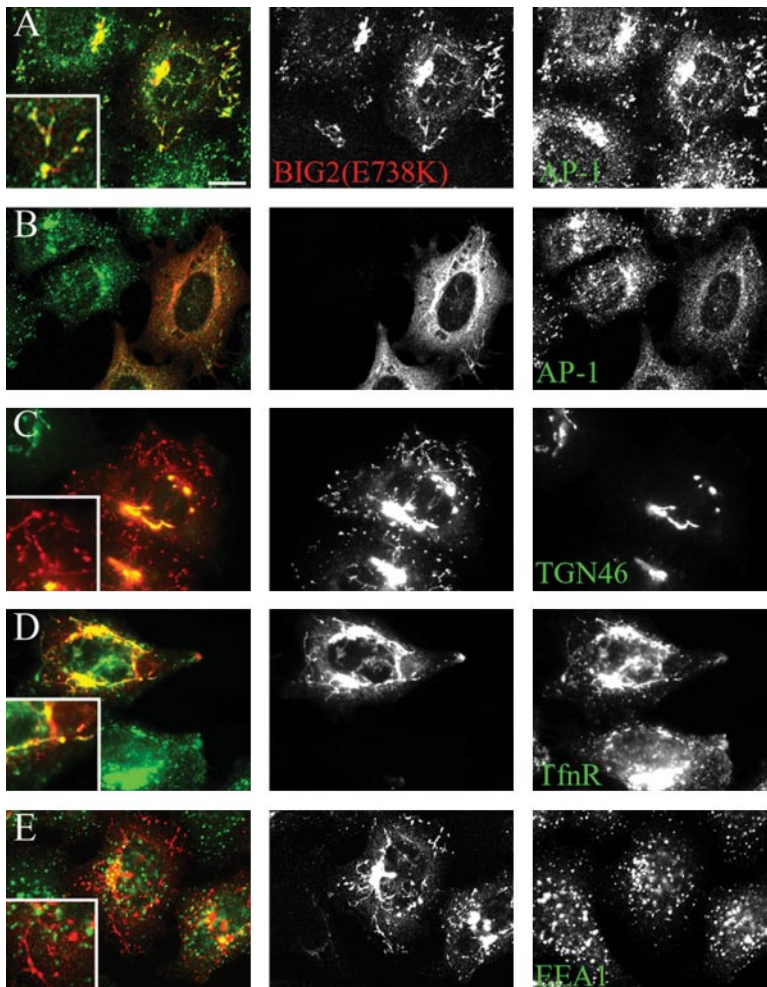

Figure 3. Immunofluorescence analysis of cells expressing BIG2(E738K). HeLa cells expressing HA-tagged BIG2(E738K) were fixed and double stained for HA and either the AP-1 γ-adaptin subunit (A and B), TGN46 (C), TfnR (D), or EEA1 (E). Bar, 10 μm.
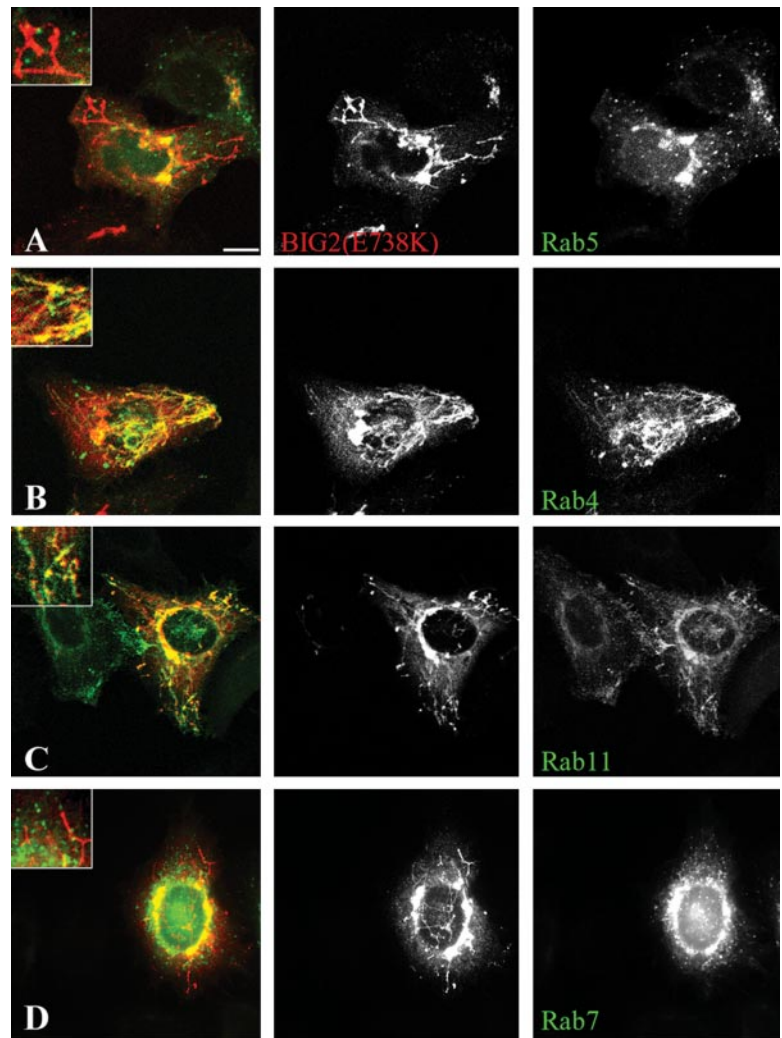
Figure 4. Localization of Rab4 and Rab11, but not Rab5 or Rab7, to the BIG2(E738K)-induced tubular structures. HeLa cells expressing HA-tagged BIG2(E738K) and either EGFP-tagged Rab5 (A) or Rab4 (B), or ECFP-tagged Rab11 (C) or Rab7 (D) were fixed and stained with anti-HA antibody. Bar, 10 μm.

(markers for the TGN), and β-COP (Supplementary Figure S1D; a *cis/medial*-Golgi coat component) remained associated with the Golgi region, and none were found on the BIG2(E738K)-positive tubular structures, indicating that neither the Golgi complex nor the TGN was disrupted by the BIG2(E738K) expression. On the other hand, AP-1 was found on the BIG2(E738K)-positive tubular structures in addition to the TGN (Figure 3A, inset). In cells with BIG2(E738K) being highly overexpressed, AP-1 was dispersed into the cytoplasm (Figure 3B), which is consistent with our previous report (Shinotsuka *et al.*, 2002a). Interestingly, in the BIG2(E738K)-expressing cells, the TfnR-positive structures also were tubulated, and the labelings for BIG2(E738K) and TfnR were found on the same tubular structures (Figure 3D, inset). In contrast, EEA1 (Figure 3E) or Lamp-1 (Supplementary Figure S1E) was not detected on the tubular structures. These observations demonstrate that the BIG2(E738K)-induced tubules are derived from the RE but not from the EE, LE, or the Golgi complex. The tubular structures were disrupted by the nocodazole treatment (our unpublished data), indicating that they were formed in a microtubule-dependent manner (Shinotsuka *et al.*, 2002b).

However, the above-mentioned observations are apparently incompatible with previous results that BFA-induced tubules from both the TGN and endosomes (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991). We therefore compared the localization of TGN46 and BIG2(WT) when cells were treated with BFA. As shown in Figure 2B, structures containing TGN46 and BIG2(WT) were indeed tubulated upon the BFA treatment. These two proteins were colocalized on some population of tubules (white arrows). However, the other population of tubules (red arrows) contained BIG2(WT) but were devoid of TGN46. These observations suggest that the BIG2-positive and TGN46-negative tubular structures induced by BFA are not originated from the TGN but may be from endosomes.

Association of Rab4 and Rab11 with the BIG2(E738K)-induced Tubular Structures

We then examined the subcellular localization of endosomal Rab proteins when coexpressed with BIG2(E738K). As shown in Figure 4, recycling endosomal Rab proteins Rab4 (Figure 4B) and Rab11 (Figure 4C) were found on the tubular structures induced by the BIG2(E738K) expression. In contrast, early endosomal Rab5 (Figure 4A) or late endosomal Rab7 (Figure 4D) was not associated with the tubules. These observations together indicate that BIG2(E738K) selectively induces tubules from the Rab4- and/or Rab11-positive endosomal compartments but not from the Rab5- or Rab7-positive one.

Internalized Tfn and Stx1 but Not EGF Are Accessible to the BIG2(E738K)-induced Tubular Compartment

Next, we examined whether internalized ligands, Tfn, Stx1, and EGF, are accessible to the BIG2(E738K)-induced tubular compartment. Tfn is internalized into cells mediated by TfnR and recycled back to the plasma membrane via the RE (Hopkins, 1983a; Dunn *et al.*, 1989); Stx1 binds to the glyco-sphingolipid Gb3 on the cell surface and is transported to the ER through the RE and the Golgi (Sandvig *et al.*, 1992; Mallard *et al.*, 1998); and EGF, after binding to EGF receptor, is transported to the lysosome via the EE and LE for degradation. In the experiments shown in Figure 5, BIG2(E738K)-expressing HeLa cells were allowed to internalize Alexa488-conjugated Tfn (Figure 5A) or EGF (Figure 5C), or Cy3-conjugated Stx1 (Figure 5B) for indicated periods of time at 37°C and then immunostained for BIG2. The BIG2(E738K) expression did not inhibit the internalization of Tfn, Stx1, or EGF. After 2.5-min uptake, Tfn was found mostly in the EE, and no morphological difference of the Tfn-containing compartments was detected between the cells with and without the BIG2(E738K) expression (Figure 5A, 2.5 min). After 5-min internalization, some population of Tfn entered the tubular structures (our unpublished data). At later time points (Figure 5A, 15 and 30 min), when Tfn reaches the Rab4/Rab11-positive RE (Sönnichsen *et al.*, 2000), the majority of Tfn signals were found on the BIG2(E738K)-positive tubules, indicating that Tfn is accessible to the tubular compartment. These observations are compatible with the previous study showing that BFA induces tubulation of the Rab4/Rab11-positive compartments that contain internalized Tfn (Sönnichsen *et al.*, 2000). After 5-min internalization, Stx1 started to enter the tubular structures close to the plasma membrane (Figure 5B, 5 min). After 20-min internalization (Figure 5B, 20 min), it was found on the BIG2(E738K)-positive tubular structures like Tfn. Tfn and Stx1 thus seem to follow the same route to the tubular compartment. After >30-min internalization (Figure 5B, 60 min), Stx1 was found not only on the tubular structures but also in the Golgi region, being compatible with the previous study showing that Stx is transported to the Golgi and in turn the ER through the RE (Mallard *et al.*, 1998). Thus, both Tfn and Stx1, which have reached the RE, can access the BIG2(E738K)-induced tubular structures. In contrast, EGF was not accessible to the tubular structures at any time point after internalization (Figure 5C). This is consistent with our observations that BIG2(E738K) induced tubulation of the Rab4- and Rab11-positive compartments but not that of Rab5- or Rab7-positive one (Figure 4). These observations suggest that the BIG2 mutant selectively induces tubulation of the RE.

Tfn recycling is marginally affected by BFA (Lippincott-Schwartz *et al.*, 1991) and moderately inhibited by Rab11(S25N) (Ullrich *et al.*, 1996; Ren *et al.*, 1998; Wilcke *et al.*, 2000). In line with these data, we observed that Tfn recycling was largely unaffected in the BIG2(E738K)-expressing cells (Figure 6). After 60-min internalization of fluorescently labeled Tfn, cells were chased for indicated periods of time in the absence of labeled Tfn. The labeled Tfn accumulated in the BIG2(E738K)-positive tubular structures was externalized out to the medium after 60 min chase (Figure 6B) as in the BIG2(WT)-expressing cells (Figure 6A). These observations indicate that the morphological change of the RE induced by BIG2(E738K) does not affect the Tfn recycling through this compartment.

Internalized TGN38 Is Accessible to the BIG2(E738K)-positive Tubular Compartment

Rat TGN38 is an orthologue of human TGN46 and known to cycle between the TGN and the plasma membrane at a very low rate (Ladinsky and Howell, 1993; Reaves *et al.*, 1993; Molloy *et al.*, 1994), although it localizes mainly to the TGN. During the retrograde transport from the plasma membrane to the TGN (Ghosh *et al.*, 1998; Mallet and Maxfield, 1999), TGN38/TGN46 passes through the RE en route to the TGN. We next examined whether internalized TGN38 enters the BIG2(E738K)-positive tubular compartments during its retrograde transport. To this end, we performed an antibody uptake experiment. Namely, exoplasmically FLAG-tagged TGN38 was coexpressed with either BIG2(WT) or BIG2(E738K) in HeLa cells, and its retrograde transport was analyzed by after extracellularly applied anti-FLAG antibody (Figure 7). In cells expressing BIG2(WT) and FLAG-TGN38, anti-FLAG antibody was found mainly on peripheral punctate structures with some population being colocalized with BIG2(WT) after 10-min internalization (Figure 7A, 10 min, inset). At later time points, the majority of the anti-FLAG antibody was transported to the TGN and colocalized with BIG2(WT) (Figure 7A, 30 and 60 min). Moreover, the antibody also was found on peripheral punctate structures positive for BIG2(WT) (Figure 7A, insets). In BIG2(E738K)-expressing cells, the anti-FLAG antibody internalized for 10 min was associated mainly with the peripheral punctate structures and some population with tubules positive for BIG2(E738K) (Figure 7B, 10 min, inset). After 30-min uptake, the anti-FLAG antibody was less found on the punctate structures but more on the BIG2(E738K)-induced tubules (Figure 7B, 30 min, inset). At later time points, the antibody was accumulated in the perinuclear region (Figure 7B, 60 min). Together, Stx1, TGN38, and Tfn, all of which transit the RE en route to the Golgi complex or plasma membrane, are accessible to the BIG2(E738K)-induced tubular compartments. Similar to the Tfn recycling, the retrograde transport of Stx1 and TGN38 from the plasma membrane via the RE to the Golgi seems not to be affected by the morphological change of the RE induced by BIG2(E738K) (see *Discussion*).

All the data presented so far are consistent with our view that BIG2 is associated not only with the TGN but also with the RE, from which BIG2(E738K) selectively induces membrane tubules.

BIG2 Is a Specific GEF toward Class I ARFs

Six ARFs (ARF1–ARF6) are present in mammals albeit humans lacking ARF2, and grouped into three classes: class I, ARF1–ARF3; class II, ARF4 and ARF5; and class III, ARF6 (Welsh *et al.*, 1994). It was shown that BIG1 purified from bovine brain has a GEF activity toward ARF1 and ARF3 (Morinaga *et al.*, 1996) and recombinant BIG2 catalyzes guanine-nucleotide exchange on ARF1, ARF5, and ARF6 in vitro (Togawa *et al.*, 1999). However, it was not addressed whether BIG2 shows the same substrate specificity in vivo. To address this issue, we made use of a pull-down assay with the GGA1-GAT domain, which is known to specifically interact with GTP-bound ARFs (Dell'Angelica *et al.*, 2000; Santy and Casanova, 2001; Shinotsuka *et al.*, 2002b; Takatsu *et al.*, 2002). Lysates prepared from HeLa cells expressing each ARF isoform alone or in combination with BIG2(WT) were applied to the GST-GGA1-GAT domain immobilized on glutathione-Sepharose beads, and the bound materials were analyzed as shown in Figure 8; we did not examine ARF4 because when expressed in cells, its expression level
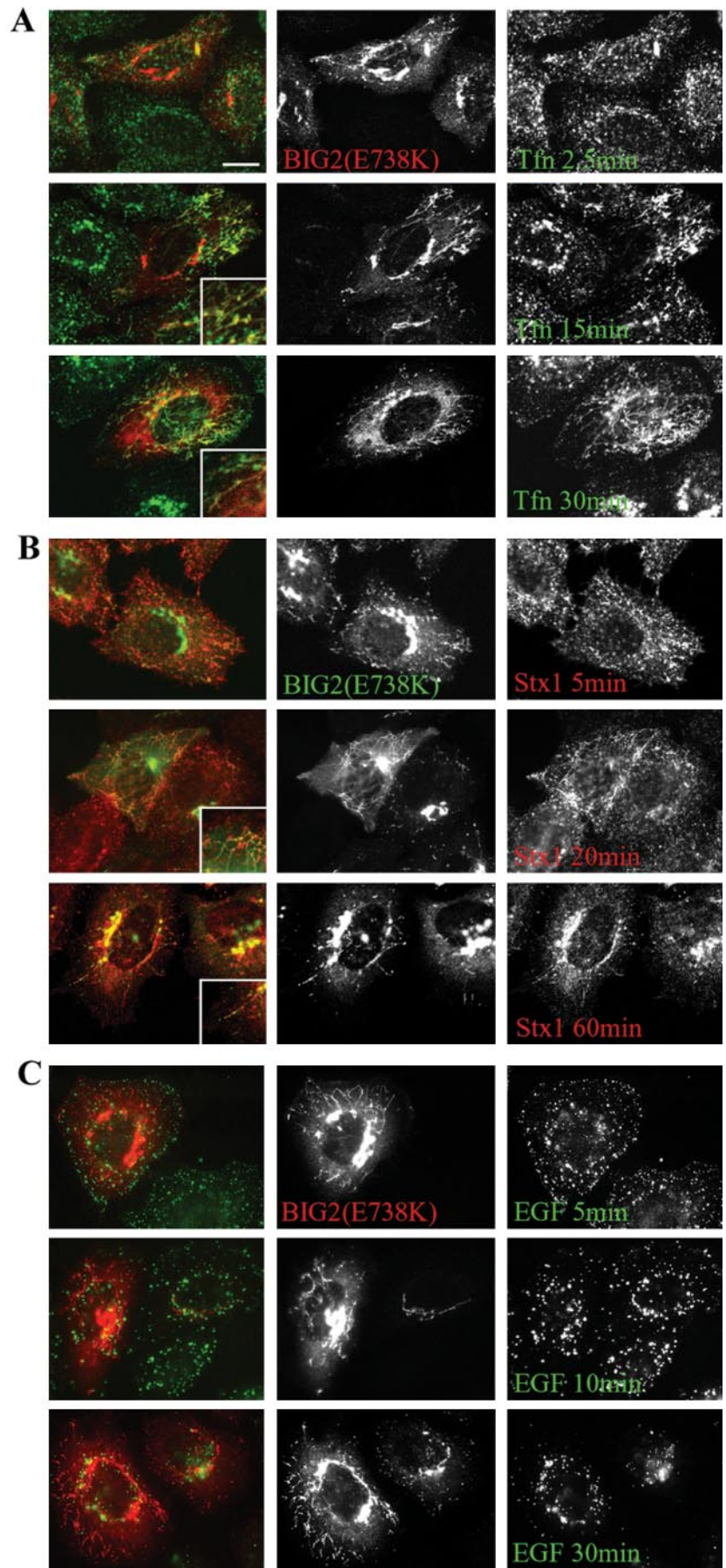
Figure 5. Incorporation of internalized Tfn and Stx1 but not EGF into the BIG2(E738K)-induced tubular structures. (A) HeLa cells expressing HA-BIG2(E738K) were serum starved for 3 h and incubated with Alexa488-conjugated Tfn at 37°C for 2.5, 5, or 30 min. (B) HeLa cells expressing HA-BIG2(E738K) were incubated with Cy3-conjugated Stx1 on ice for 50 min, washed, and incubated at 37°C for 5, 20, or 60 min. (C) HeLa cells expressing HA-BIG2(E738K) were serum starved for 3 h and incubated with Alexa488-conjugated EGF at 37°C for 5, 10, or 30 min. The cells were then fixed and stained with anti-HA antibody followed by Cy3-conjugated (A and C) or Alexa488-conjugated (B) anti-rat secondary antibodies. Bar, 10 μm.
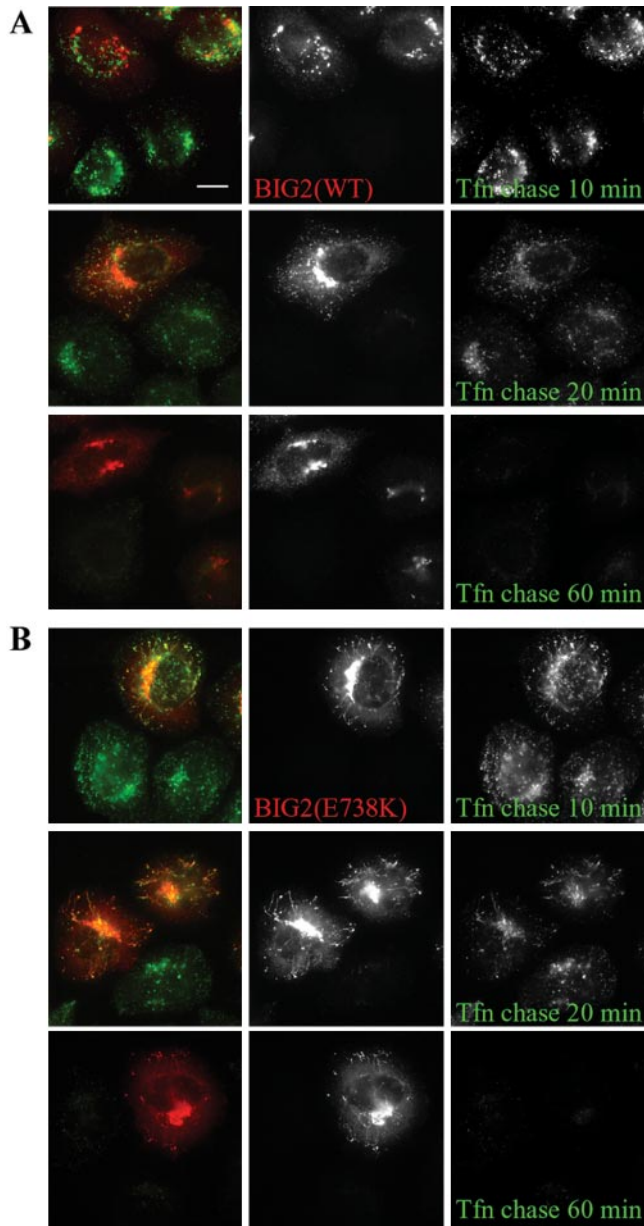
Figure 6. Tfn recycling is not affected by the BIG2 (E738K) expression. HeLa cells expressing HA-tagged BIG2(WT) (A) or BIG2(E738K) (B) were serum starved for 3 h and incubated with Alexa488-conjugated Tfn at 37°C for 60 min. The cells were washed extensively and incubated with medium that did not contain labeled Tfn at 37°C for indicated periods of time. Bar, 10 μm.

Figure 7. Internalized TGN38 enters the BIG2(E738K)-induced tubular structures. HeLa cells coexpressing FLAG-TGN38 and either HA-tagged BIG2(WT) (A) or BIG2(E738K) (B) were incubated with anti-FLAG M2 antibody on ice for 50 min, washed, and further incubated at 37°C for 10, 30, or 60 min. Bar, 10 μm.

was extremely low compared with those of other ARFs for unknown reasons. More ARF1 and ARF3 in the BIG2-coexpressing cells bound to the GGA1-GAT domain than those in cells without exogenous BIG2 expression (Figure 8A, bottom; compare lanes 2 and 3, and lanes 4 and 5, respectively). By quantifying the band intensity, approximately three- and fourfold larger amounts of GTP-bound ARF1 and ARF3, respectively, were estimated to be present in the BIG2-overexpressing cells than those in the control cells (Figure 8B). In contrast, only 1.5-fold larger amounts of ARF5 were bound to GGA1-GAT and the ARF6 amount bound to GGA1-GAT did not change by the BIG2 overex-
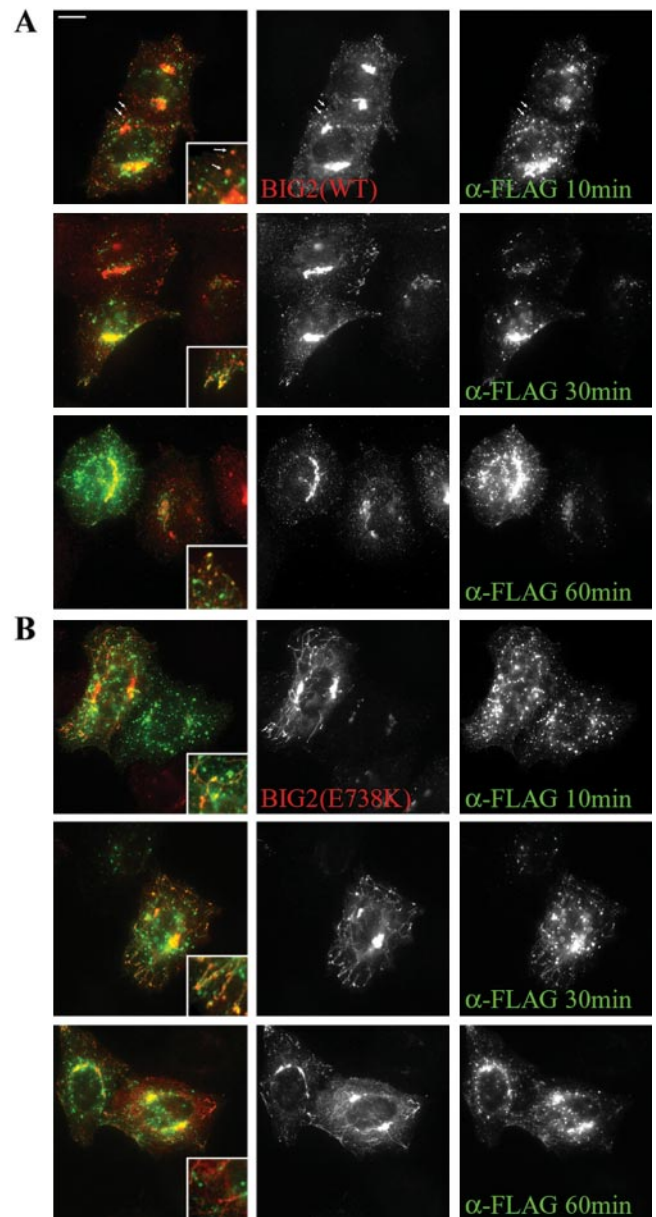
pression (Figure 8B). These results suggest that BIG2 specifically activates class I ARFs (ARF1 and ARF3) in vivo.

Coexpression of a Dominant-Negative Mutant of Class I ARF Promotes the BIG2(E738K)-induced Tubulation

We next asked whether ARF is related to the BIG2(E738K)-mediated tubule formation. To this end, we examined whether coexpression of dominant-negative ARF mutants ARF1(T31N) and ARF3(T31N) affects the BIG2(E738K)-induced tubulation. As shown in Figure 9, coexpression of ARF1(T31N) (Figure 9A) or ARF3(T31N) (Figure 9B) extremely accentuated the BIG2(E738K)-induced tubulation. In striking contrast, coexpression of ARF5(T31N) (Figure 9C)
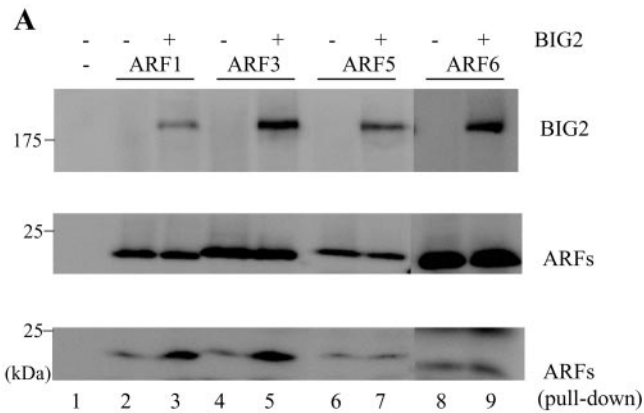
Figure 8. BIG2 has a GEF activity toward ARF1 and ARF3 in vivo. (A) HeLa cells were transfected with the indicated expression vectors and then lysed. The cell lysates ($\sim$500 μg of protein) were subjected to pull-down assay with the GST-GGA1-GAT domain as described under *Materials and Methods* (bottom). Top and middle, expression levels of HA-BIG2 and ARF-HA, respectively, by subjecting the cell lysates directly to Western blot analysis by using anti-HA antibody. (B) Fold activation of each ARF by coexpressed BIG2. The ratios of bound versus total ARFs were estimated from the band intensities in the middle and bottom panels in A. This is the representative of three independent experiments.

Figure 9. Dominant-negative mutants of ARF1 and ARF3 enhance the BIG2(E738K)-induced tabulation. HeLa cells coexpressing untagged BIG2(E738K) and either HA-tagged ARF1(T31N) (A), ARF3(T31N) (B), ARF5(T31N) (C), or ARF6(T27N) (D) were fixed and double stained with polyclonal rabbit anti-BIG2 and monoclonal rat anti-HA antibodies followed by Cy3-conjugated anti-rabbit and Alexa488-conjugated anti-rat secondary antibodies. In C, red arrows indicate the shortened tubules. Bar, 10 μm.

or ARF6(T27N) (Figure 9D) did not significantly promote the BIG2(E738K)-induced tubule formation; rather, the ARF5(T31N) expression showed a tendency to shorten the tubules (red arrows; see *Discussion*). These observations suggest that BIG2(E738K)-induced tubulation is ascribed to the inactivation of ARF1 and ARF3 and that expression of BIG2(E738K) is not enough to inactivate all class I ARF present in the cells, probably because of the presence of endogenous BIG2 and/or another GEF such as BIG1. Together, it seems likely that class I ARFs associated with the RE are implicated in the structural integrity, but not the function, of this compartment.

DISCUSSION

The Gea/GBF/GNOM and Sec7/BIG subfamilies of ARF-GEFs are present in specific cellular locations and regulate various aspects of membrane trafficking by activating distinct classes of ARFs. Here, we have extended our previous studies (Shinotsuka *et al.*, 2002a,b) and made four important
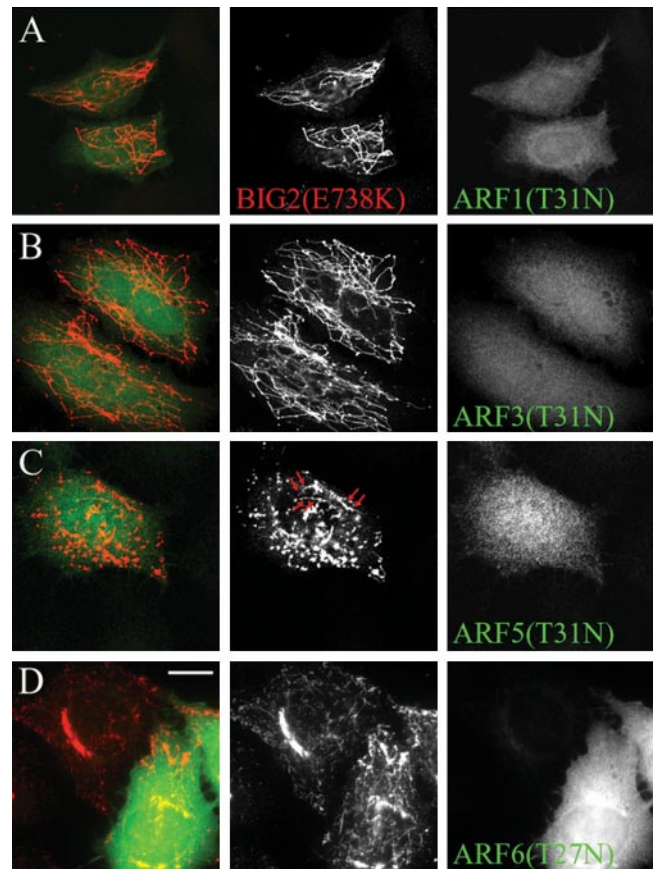
findings concerning BIG2. First, BIG2 is associated with the TfnR- and Rab4/Rab11-positive RE as well as with the TGN. Second, expression of a catalytically inactive mutant of BIG2, E738K, selectively induces membrane tubules from the RE. Third, despite the dramatic morphological change of the RE, neither internalization nor recycling of Tfn nor retrograde transport through the RE of Stx1 or TGN38 was apparently affected by the BIG2(E738K) expression. Finally, BIG2 shows a GEF activity toward class I ARFs (ARF1 and ARF3) in vivo and coexpression of the dominant-negative ARF1 or ARF3 mutant makes the BIG2(E738K)-induced membrane tubules extremely prominent.

We and the laboratory of Moss and Vaughan previously showed that BIG2 localizes mainly to the TGN (Yamaji *et al.*, 2000; Shinotsuka *et al.*, 2002a,b). At that time, however, both groups noticed that BIG2 also is found on punctate structures distributed throughout the cytoplasm. Moreover, GNOM (one of BFA-sensitive ARF-GEFs in *Arabidopsis*) has recently been shown to localize to endosomes and is involved in the recycling of plasma membrane proteins from the endosomes (Geldner *et al.*, 2003). In the present study, we have revealed that the punctate structures containing BIG2 correspond to the RE positive for AP-1, TfnR, and Rab4/Rab11. Furthermore, we have shown that when ex-

pressed in cells, BIG2(E738K) induces membrane tubules from the RE but not from the EE, LE, or the TGN and that BIG2(E738K) itself is associated with the tubules.

Sönnichsen *et al.* (2000) previously revealed the organization of membrane domains of endosomes positive for distinct Rab proteins by following internalized Tfn. They found that Tfn was transported sequentially to Rab5-, Rab5/Rab4-, and Rab4/Rab11-positive endosomes and that, interestingly, only the membrane domains on the Rab4/Rab11-positive endosomes were sensitive to BFA. These data are in line with our observations that BIG2(E738K) induces membrane tubules from the Rab4/Rab11-positive endosomes but not from Rab5- or Rab7-positive ones (Figure 4).

Treatment of cells with BFA caused tubulation of compartments containing BIG2(WT) and TGN46. Although some population of the BFA-induced tubules contained both proteins, another population contained only BIG2(WT) (Figure 2). These observations suggest that BIG2(WT)-positive and TGN46-negative tubules are not derived from the TGN. In line with this speculation, the membrane tubules induced by BIG2(E738K) contained the BIG2 mutant and markers for the RE but not TGN46. A straightforward explanation for these observations is that BFA induces membrane tubules from both the TGN and RE, whereas BIG2(E738K) induces them only from the RE. It is tempting to speculate that tubulation of the TGN induced by BFA results from inhibition of another BFA-sensitive ARF-GEF (e.g., BIG1).

We have shown that BIG2 has a GEF activity toward class I ARFs in vivo (Figure 8) and that overexpression of the dominant-negative mutants of class I ARFs synergistically enhances the BIG2(E738K)-induced membrane tubulation (Figure 9). It therefore seems likely that a population of class I ARFs is associated with the RE and implicated in the structural integrity of this compartment. Transport carriers are formed by a sequence of events, including cargo loading, coat assembly, membrane budding, and fission. We envisage that the tubules resulted from the inhibition of the budding and/or fission of membranes at the RE that involves BIG2 and class I ARFs. In support of this view, the Rab11(S25N)-induced tubulation of Tfn-positive endosomal structures seemed to be partially suppressed by coexpression of BIG2(WT), which can activate endogenous ARF1 and ARF3 (compare Figure 2A and Supplementary Figure S2).

In contrast to the class I ARF mutants, neither the ARF5 nor ARF6 mutant accentuated the BIG2(E738K)-induced tubulation. ARF5(T31N), rather, had a tendency to suppress the tubulation, although we did not further address the significance of this phenomenon in the present study.

Consistent with our previous study (Shinotsuka *et al.*, 2002b), the BIG2(E738K) expression altered the AP-1–positive endosomal structures (Figure 2), suggesting that BIG2 is involved in an AP-1–dependent trafficking process. The AP-1 complex plays a key role in the formation of clathrin-coated vesicles that carry cargoes between the TGN and endosomes, although it is currently a matter of debate on the direction of the AP-1/clathrin-mediated transport: antero-grade, retrograde, or both (Meyer *et al.*, 2000; Rohn *et al.*, 2000). AP-1 also has been shown to be involved in recycling back of TfnR to the cell surface via the RE (Stoorvogel *et al.*, 1996; Futter *et al.*, 1998). Furthermore, the immunoelectron microscopic studies have shown that the AP-1 is associated with Tfn- and Stx-containing membrane domains of the RE, namely, one domain responsible for recycling back of proteins to the plasma membrane and the other for retrograde transport to the TGN, respectively. It is tempting to speculate that BIG2 recruits the AP-1 complex onto the RE through activating class I ARFs, although we do not know

on which membrane domains BIG2 functions. We and others previously reported that the ear domain of γ1-adaptin, a subunit of the AP-1 complex, binds to Rabaptin-5, which is a dual effector of Rab5 and Rab4 (Hirst *et al.*, 2000; Shiba *et al.*, 2002). Moreover, it was recently demonstrated that Rab4-GTP acts as a scaffold for a Rabaptin-5-γ1-adaptin complex on the recycling endosomal membranes, whereas Rab5 does not have such a role (Deneka *et al.*, 2003). Altogether, it is likely that BIG2 activates class I ARFs in the recruitment of AP-1 onto endosomal membranes and Rab4 and Rabaptin-5 may specify and/or stabilize its location on the endosomal compartments.

Despite the dramatic morphological change of the RE induced by the BIG2(E738K) expression, both internalization and recycling of Tfn and transit of Stx1 and TGN38 through this compartment seem to be largely unaffected (Figure 5). In agreement with this, BFA does not significantly inhibit endocytic uptake or trafficking through endocytic compartments (Lippincott-Schwartz *et al.*, 1990; Damke *et al.*, 1991; Wood and Brown, 1992; Reaves *et al.*, 1993), unlike its effects on the secretory pathway. However, we cannot exclude a possibility that BIG2 may have a role in the sorting of Tfn on the RE in polarized cells, in which more precise sorting events are required than that in nonpolarized cells. In fact, BFA treatment of polarized Madin-Darby canine kidney cells affects the basolateral sorting of Tfn, whereas BFA does not significantly affect its recycling (Futter *et al.*, 1998).

Our finding that, in conjunction with class I ARFs, BIG2 is implicated in the structural integrity of the RE will promote investigation of the molecular mechanism underlying cross talk between the biosynthetic and endocytic/recycling pathways. Further dissection of the functions of the BIG family proteins will provide insights into where ARFs are activated, trigger assembly of appropriate coat components and recruit or activate its downstream effectors to control the budding/fission or sorting processes.

ACKNOWLEDGMENTS

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