ADP-ribosylation factors (ARFs) and their activating guanine nucleotide exchange factors (GEFs) play key roles in membrane traffic and signaling. All ARF GEFs share a ∼200-residue Sec7 domain (Sec7d) that alone catalyzes the GDP to GTP exchange that activates ARF. We determined the crystal structure of human BIG2 Sec7d. A C-terminal loop immediately following helix J (loop J) was predicted to form contacts with helix H and the switch I region of the cognate ARF, suggesting that loop J may participate in the catalytic reaction. Indeed, we identified multiple alanine substitutions within loop J of the full length and/or Sec7d of two large brefeldin A-sensitive GEFs (GBF1 and BIG2) and one small brefeldin A-resistant GEF (ARNO) that abrogated binding of ARF and a single alanine substitution that allowed ARF binding but inhibited GDP to GTP exchange. Loop J sequences are highly conserved, suggesting that loop J plays a crucial role in the catalytic activity of all ARF GEFs. Using GEF mutants unable to bind ARF, we showed that GEFs associate with membranes independently of ARF and catalyze ARF activation in vivo only when membrane-associated. Our structural, cellular biological, and biochemical findings identify loop J as a key regulatory motif essential for ARF binding and GDP to GTP exchange by GEFs and provide evidence for the requirement of membrane association during GEF activity.

ADP-ribosylation factors (ARFs) are key regulators of membrane traffic and intracellular signaling. ARFs cycle between an inactive GDP-bound state and an active GTP-bound state. The activation occurs by a GDP to GTP exchange mediated by guanine nucleotide exchange factors (GEFs) characterized by a conserved Sec7 domain (Sec7d). Mammalian Sec7d GEFs are divided into five classes based on size and sequence similarities. The large (>200-kDa) GEFs include GBF1, BIG1, and BIG2. The small (<200-kDa) GEFs include the BRAG, EFA6, ARNO/cytohesin/Grp, and F-box subfamilies (1). The division also reflects the sensitivity or resistance to brefeldin A (BFA). The large GEFs are BFA-sensitive, whereas the small GEFs are BFA-resistant.

The Sec7d alone mediates GDP to GTP exchange on ARF in vitro (2, 3). Structural studies of ARNO Sec7d show an elongated protein composed of 10 α-helices (A–J) forming a compact rod-shaped structure with a hydrophobic groove (4, 5). The groove includes α-helix H and a loop between α-helices F and G and forms the interface for ARF binding. As expected based on conservation of protein–protein interfaces, these domains are highly conserved in various GEF subfamilies and among different species (6) (see Fig. 1B).

Mutations R152V, G155N, E156K, Q158A, M194K (4), and N201A (5) (numbering based on ARNO Sec7d) within the Sec7d groove have identified residues critical for GDP to GTP exchange. The invariant Glu-156 “glutamic finger” within the F-G loop is essential for the destabilization of the Mg<sup>2+</sup> and GDP from ARF1, and Sec7d with mutations in Glu-156 form a complex with ARF-GDP but do not catalyze the release of GDP (7). Despite extensive study of Sec7d-mediated ARF activation, the contribution of motifs outside the F–H region is largely unknown. Identifying all motifs required for ARF activation is necessary to understand all inputs that regulate GEF activity and is essential for the rational design of GEF-specific inhibitors.

Phosphorylation of BIG1 on Ser-883 that lies proximal to the J helix has been shown to decrease BIG1 catalytic activity (8). Interestingly, the analogous region within ARNO Sec7d folds (thio)triphosphate; ER, endoplasmic reticulum; ERES, ER exit sites; TGN, trans-Golgi network; RE, recycling endosome; TfnR, transferrin receptor; AP, adaptor protein.
into a loop from Glu-241 to Pro-246 and interacts tightly with α-helices F, H, and J by packing Phe-243 between Ile-238 and Leu-148 and by packing Pro-242 against His-200 (5). Together, these results suggest that the loop immediately following helix J (loop>J) might participate in ARF binding and/or GDP to GTP exchange.

We determined the crystal structure of BIG2 Sec7d and show that the C-terminal residues within loop>J contact helices F and H and possibly the switch I region of the cognate ARF. We interrogated the functional significance of loop>J by generating alanine substitutions within this motif in the large BFA-sensitive GBF1 and BIG2 and the small BFA-resistant ARNO. The critical role of loop>J in GEF function was explored in vivo and in vitro. Our structural, cellular, and biochemical findings indicate that loop>J forms part of the substrate-binding pocket for ARF and is essential for the catalytic activity of ARF GEFs. Our findings that GEFs defective in ARF binding have dominant negative effects in cells have significant implications for the mechanism by which GEFs catalyze ARF activation in vivo.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and Plasmids—Rabbit anti-GBF1 antibodies have been described (9). Mouse monoclonal anti-BIG2 antibodies were made at The Epitope Recognition and Immuno-reagent Core Facility (University of Alabama, Birmingham, AL). The following antibodies were commercially obtained: monoclonal anti-API from Sigma-Aldrich, monoclonal anti-GBF1 from BD Transduction Laboratories, polyclonal and monoclonal anti-GFP from Abcam (Cambridge, MA), polyclonal anti-β-COP and monoclonal anti-GM130 from Affinity Bioreagents (Golden, CO), polyclonal anti-giantin from Covance (Princeton, NJ), monoclonal anti-transferrin receptor from Zymed Laboratories Inc. (South San Francisco, CA), and monoclonal anti-HA from Roche Applied Science. Secondary antibodies conjugated with HRP, Alexa Fluor 488, or Alexa Fluor 594 were from Molecular Probes (Eugene, OR). BFA was from Sigma-Aldrich.

GBF1 cDNA has been described (9). GFP-tagged wild-type GBF1 was constructed by subcloning GBF1 into the pEGFP vector using XhoI and Xmal restriction enzymes. This results in a GFP extension at the N terminus of GBF1. GFP-tagged GBF1/E794K has been described (9). ARF1-T31N-HA, construct was a gift from Dr. Julie Donaldson (National Institutes of Health, Bethesda, MD). HA-tagged wild-type BIG2 and BIG2/E738K were a gift from Dr. Kazu Nakayama (University of Tsukuba, Ibaraki, Japan). FLAG-tagged wild-type ARNO and ARNO/E156K were a gift from Dr. James Casanova (University of Virginia, Charlottesville, VA). GBF1/7A, BIG2/3A, and ARNO/1A were generated by PCR-based mutagenesis. The Sec7 domains of BIG2, GBF1, and ARNO were fused in-frame to glutathione S-transferase in pGEX5X-3 (Amersham Biosciences).

Sample Production for X-ray Crystallography—Samples were prepared by the standard process of the Northeast Structural Genomics Consortium (10, 11). A construct corresponding to residues 635–836 of Sec7d of human argef2 gene (Northeast Structural Genomics identification number HR5562A) was cloned into a modified pET14 expression vector (Novagen) containing an N-terminal affinity tag (MGHHHHHHSSH), yielding the plasmid HR5562A-14.13 (available from the Protein Structure Initiative Materials Repository). The HR5562A-14.13 plasmid was transformed into codon-enhanced BL21(DE3) pMGK Escherichia coli and cultured in M9 minimal medium containing selenomethionine, lysine, phenylalanine, threonine, isoleucine, leucine, and valine and for SeMet labeling as described (11). Initial bacterial growth was carried out at 37 °C, and protein expression was induced at 17 °C with 1 mM isopropyl β-d-thiogalactopyranoside. Expressed proteins were purified using an ÄKTAxpress™ (GE Healthcare) two-step protocol consisting of HisTrap HP affinity chromatography followed directly by HiLoad 26/60 Superdex 75 gel filtration chromatography. The final yield of purified isotopically enriched Sec7d was ~18 mg/ml of culture. Analytical gel filtration chromatography and static light scattering data showed the protein to be homogeneous (>95%) and monomeric in solution under neutral buffer conditions (100 mM Tris, 100 mM NaCl, 250 ppm NaN3, pH 7.5).

Crystallography and Data Collection—Two microliters of selenomethionine-substituted Sec7d were mixed in with 2 μl of mother liquor containing 15% (w/v) PEG 1000 and 0.1 M sodium citrate, pH 5.0. Crystals were grown at 18 °C by the hanging drop vapor diffusion method, cryoprotected with 20% (v/v) glycerol, and flash frozen in liquid nitrogen. Several diffraction data sets were collected at the beamlines X4A, X4C, and X6A of the National Synchrotron Light Source at Brookhaven National Laboratory. Data were processed with the HKL2000 package (12). The crystals belong to P2_1 space group with unit cell parameters a = b = 53.9 Å and c = 75.7 Å. There is one monomer per asymmetric unit. Residues beyond Ala-828 are disordered in the current structure.

Structure Determination and Refinement—Sec7d structure was solved by the SAD method with SHELX (13). The locations of seven selenium sites were identified from a 3.0-Å data set. After solvent flipping, a σA-weighted Fourier summation yielded an interpretable map. After phase refinement, an initial model constructed with Resolve (14) was refined with CNS (15). Manual model building was performed using Coot (16). Several cycles of simulated annealing and minimization were carried out using CNS (15). The crystal was twinned with twinning fraction 0.32. The twinning parameters were used in the CNS refinement. The crystallographic statistics for data collection and refinement are summarized in Table 1. Protein coordinates have been deposited in the Protein Data Bank (code 3L8N).

Mammalian Cell Culture and Transfection—HeLa cells were grown as described (17) and transfected with Mirus TransIT-

| TABLE 1
| C-terminal Regulatory Motif in Sec7 Domain |
|-----------------------------------------|
| Summary of crystallographic information |
| Values in parentheses are for the highest resolution shell. r.m.s.d., root mean square deviation. |
| Resolution (Å) | 50.30 (3.1-3.0) |
| Unique reflections | 9252 |
| Mean I/σI | 14.6 (2.3) |
| Completeness | 95 (99) |
| Redundancy | 2.2 (2.4) |
| Rmerge (0.309) | 0.069 |
| R | 0.203 |
| Free R | 0.262 |
| r.m.s.d. | |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 1.3 |
C-terminal Regulatory Motif in Sec7 Domain

LT1 Transfection Reagent (Mirus Bio Corp., Madison, WI). 24 h after transfection, cells were processed for immunoflou- 
rescence (IF) or lysed with immune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% 
sodium deoxycholate, 0.1% SDS). Some cells were treated with 
5 μg/ml BFA for 1 h and then processed for IF or washed 
with PBS and incubated with fresh medium without BFA for 2 h 
prior to IF.

Microscopy—Cells were processed for IF as described (17) 
and visualized with a Leitz Orthoplan microscope with epifluo-
rescence and Hoffman Modulation Contrast optics from 
Chroma Technology, Inc. (Brattleboro, VT). Images were 
captured with a charge-coupled device high resolution camera 
from Roper Scientific (Tucson, AZ) equipped with a camera/ 
computer interface and analyzed using IPLab Spectrum soft-
ware (Scanalytics Inc., Fairfax, VA). Live cell fluorescence 
imaged is indicated by 

RESULTS

Crystal Structure of Human BIG2 Sec7d—The structure of 
BIG2 Sec7d was determined at 3.0-Å resolution, and a sum-
mary of crystallographic information is presented in Table 1. 
The final refined model has 188 amino acids encompassing res-
ides 637–828. Two residues at the N terminus, eight residues 
at the C terminus, and five residues in the loop regions were 
disordered, and they were not included in the atomic model. 
The overall structure shows an elongated cylinder-shaped 
assembly of 10 helices (Fig. 1C). The ARF-binding hydrophobic 
groove is formed by helices F, G, and H and is solvent-exposed 
in the current structure. The loop between F and G helices 
carries the Glu-738 that is essential for GDP to GTP exchange.

A model of BIG2 Sec7d-ARF1 complex was constructed 
based on the solved structure of the Gea1 Sec7d-ARF1 complex 
(4, 21) by overlaying the BIG2 Sec7d with the Gea1 Sec7d (Fig. 
1D). The model suggests close contacts between the switch I 
region of ARF and helix H and the loop between helices F and G 
of BIG2 Sec7d that parallel the contacts observed with Gea1 
Sec7d. In addition, the BIG2 model suggests that residues at the 
C terminus of the Sec7d after the J helix (loop=>) are located 
close to the switch I region of ARF. In particular, Ile-827 in 
loop=> forms part of a hydrophobic cluster that also includes 
the side chains of Leu-726 and Leu-730 from helix F of BIG2 
and the side chain of Ile-49 of ARF (Fig. 1B). An analogous 
isooleucine residue in the loop=> region of Gea1 Sec7d also is 
located in a hydrophobic pocket with ARF1 (Fig. 1D).

Interestingly, Ile-827 is highly conserved in GBF1, BIG2, 
and BIG1 (Fig. 1B), suggesting an important functional role. The 
isooleucine is replaced with phenylalanine 243 in ARNO Sec7d 
and appears to form a similar hydrophobic interaction with 
ARF within the reported structure of ARNO Sec7d-ARF com-
plex (4, 5). The apposition of loop=> to ARF suggested that the 
C-terminal loop=> of Sec7d may contribute to ARF binding, 
a finding that has not been appreciated or tested before. We 
interrogated the functional significance of loop=> by introduc-
ing progressively smaller alanine substitutions within this 
region in GBF1, BIG2, and ARNO. Our rationale was to define 
the smallest region of the loop=> that is functionally relevant. 
Thus, we started by disrupting the entire loop=> (seven amino 
acids changed to alanines), then disrupting only half the loop=> 
(three amino acids changed to alanines), and finally disrupting 
only a single amino acid within loop=>. We disrupted the entire 
loop=> in GBF1 (EIVMPEE at positions 883–889) to generate 
GBF1/7A (Fig. 1B, shaded in red). A less disruptive change 
was introduced in GBF1 (EIV at positions 883–885) to generate 
GBF1/3A (Fig. 1B, shaded in yellow). We moderately disrupted 
BIG2 by changing three amino acids to alanines (KIAM at posi-
tions 826–829) to generate BIG2/3A (Fig. 1B, shaded in red). 
Finally, we exchanged a single Phe at position 243 in ARNO 
to generate ARNO/1A (Fig. 1B, shaded in red). The functions 
of full-length GEFs with these mutations and/or of the Sec7 
domains from these GEFs were characterized.

GBF1 with Mutations in Loop=>J Disrupts Golgi Architecture—
GBF1 is essential for Golgi biogenesis, and the Golgi collapses 
and Golgi proteins relocate to the ER or to punctate structures 
adjacent to ER exit sites (ERES) in cells transfected with the
catalytically inactive GBF1/E794K mutant, depleted of GBF1 by siRNA, or treated with the GBF1 inhibitors BFA and golgicide (9, 17, 22). We assessed the role of loop loop/H11022 in GBF1 function by comparing Golgi architecture in cells expressing wild-type GFP-GBF1, GFP-GBF1/E794K, or GFP-GBF1/7A. All constructs encode the correct molecular weight protein when transfected into HeLa cells (supplemental Fig. S1A). The level of GBF1/7A appeared slightly reduced, and this may reflect lower transfection efficiency or possibly increased degradation of this mutant.

FIGURE 1. Crystal structure of human BIG2 Sec7d. A schematic of GBF1, BIG2, and ARNO showing domain arrangement. DCB, dimerization cyclophilin-binding domain; HUS, homology upstream of Sec7d; HDS1–3, homology downstream of Sec7d; CC, coiled coil domain; PH, pleckstrin homology domain. B, alignment of Sec7d from GBF1, BIG2, and ARNO shows sequence conservation in the catalytic F-G loop including the invariant E (boxed in black). The α-helices are marked (A–J). Mutations in loop J in each Sec7d are boxed and highlighted in red. The loop J of BIG1 is also shown. Phosphorylation of Ser-883 (boxed and highlighted in blue) inhibits BIG1 exchange activity (8). C, a schematic drawing of the Sec7 domain of human BIG2. The 10 α-helices are labeled A through J, overlay of the BIG 2 Sec7 domain (in green) and the complex of Gea1 Sec7 domain (in yellow) and ARF1 (in cyan). GDP and BFA are shown as stick models. Mg2+ is shown as a sphere. Sw. I, switch I region of ARF1; Sw. II, switch II region of ARF1. D, detailed representation of the interactions between the C-terminal loop J of the Sec7 domain (BIG2 in green and Gea1 in yellow) with the rest of Sec7 and the switch I region of ARF1 (in cyan). The Lys-826 and Ile-827 residues within loop J make contacts with the H helix and with ARF, respectively.
Expression of GFP-GBF1/7A disrupts Golgi morphology. HeLa cells transfected with GFP-GBF1, the catalytically inactive GFP-GBF1/E794K, or the GFP-GBF1/7A mutant were processed for IF using anti-GFP and anti-giantin (gtn) antibodies. Transfected cells are marked with asterisks. Expression of GFP-GBF1/E794K and GFP-GBF1/7A disrupts Golgi architecture.

Analysis of 30 cells expressing GFP-GBF1 showed that all contain characteristic Golgi ribbon marked with the medial Golgi protein giantin (a representative cell is shown in Fig. 2A). GFP-GBF1 localized to the Golgi in a pattern analogous to that of endogenous GBF1 (not shown). In contrast, in cells expressing GFP-GBF1/E794K, giantin relocated to a diffuse ER pattern that reflects the collapse of the Golgi (Fig. 2B). Analysis of 22 cells expressing GFP-GBF1/E794K indicated that 16 (73%) have disrupted Golgi. GFP-GBF1/E794K localized in punctate structures adjacent to ERES. Cells expressing GFP-GBF1/7A also showed giantin in a diffuse pattern and in dispersed puncta scattered around the nucleus (Fig. 2C). The Golgi dispersion caused by GFP-GBF1/7A was less severe than that caused by GFP-GBF1/E794K, and only six of 29 analyzed cells (33%) had disrupted Golgi. GFP-GBF1/7A localized to the dispersed elements. The Golgi disruption caused by expression of GFP-GBF1/7A suggested that like GFP-GBF1/E794K the GFP-GBF1/7A mutant might be catalytically compromised. Analogous results were obtained with GFP-GBF1/3A (not shown), indicating that mutating only residues 883–885 (Fig. 1C) also reduces GBF1 activity.

GBF1-mediated ARF activation is essential for the recruitment of the heptameric COP1 coat to the Golgi and the ER-Golgi intermediate compartment (9, 17, 22). Expression of GFP-GBF1 had no detectable effect on COP1 recruitment as assessed by the localization of β-COP (supplemental Fig. S1C). In contrast, cells expressing the inactive GFP-GBF1/E794K showed diffuse cellular distribution of β-COP. GFP-GBF1/7A also caused dissociation of β-COP from membranes, suggesting that GFP-GBF1/7A is catalytically compromised in vivo.

GBF1 with Mutations in Loop>Y Prevents de Novo Golgi Biogenesis—The non-competitive inhibitor BFA is inserted within the ARF-GEF interface and inhibits GDP to GTP exchange (21, 23) (Fig. 1D). In cells treated with BFA, the Golgi collapses, and Golgi proteins relocate to the ER or to peripheral ERES (24). The BFA effect is reversible, and Golgi reform after BFA removal. GBF1 is required for the de novo Golgi biogenesis during BFA washout (17). The ability of GFP-GBF1/7A to sustain Golgi biogenesis was assessed by comparing Golgi reassembly in cells expressing GFP-GBF1, GFP-GBF1/E794K, or GFP-GBF1/7A. GM130 staining was used to monitor Golgi reassembly.

Cells expressing GFP-GBF1 showed typical Golgi architecture (Fig. 3A). BFA treatment caused Golgi disruption and the relocation of GM130 to ERES dispersed throughout the cell and resulted in GFP-GBF1 relocation to the ER. During a subsequent BFA washout, the Golgi reformed, and GM130 and GFP-GBF1 again localized to the Golgi. Cells expressing GFP-GBF1/E794K had collapsed Golgi with GM130 and GFP-GBF1/E794K redistributing to peripheral ERES (Fig. 3B). After BFA treatment, GM130 remained in ERES, whereas GFP-GBF1/E794K relocated to the ER. Thus, in the presence of BFA, GFP-GBF1/E794K behaves like GFP-GBF1. This is expected because both GBF1 and GFP-GBF1/E794K bind ARF to create the BFA-binding interface. During a subsequent BFA washout, the Golgi ribbon did not reform in cells expressing GFP-GBF1/E794K. GM130 remained in ERES, and GFP-GBF1/E794K moved out of the ER to co-localize with GM130. Cells expressing GFP-GBF1/7A contained collapsed Golgi with GM130 and GFP-GBF1/7A co-localizing in dispersed ERES (Fig. 3C). After BFA treatment, GM130 remained in ERES. Interestingly, a significant fraction of GFP-GBF1/7A remained co-localized with GM130 in ERES and did not relocate to the ER. This behavior differs from that of endogenous GBF1, exogenously expressed GFP-GBF1, and the GFP-GBF1/E794K mutant that were redistributed to the ER in the presence of BFA. During a subsequent BFA washout in cells expressing GFP-GBF1/7A, the Golgi ribbon did not reform, and GM130 remained in ERES where it co-localized with GFP-GBF1/7A. Our findings indicate that GFP-GBF1/7A like the inactive GFP-GBF1/E794K does not support Golgi biogenesis. This suggests that loop>Y positioned ~90 amino acids distal to the Glu–794 glutamic finger might regulate catalytic activity of GBF1.

BIG2 with Mutations in Loop>Y Causes trans-Golgi Network (TGN)/Endosomal Tubulation—BIG2 localizes to the TGN and to a lesser extent to recycling endosomes (REs) and regulates the architecture of these compartments (25). Expressing the catalytically inactive BIG2/E738K causes tubulation of TGN (marked with mannose 6-phosphate receptor, GGA1, and γ-subunit of the AP1 adaptor complex) and RE (marked with transferrin receptor (TfnR), Rab4, and Rab11) (25). To assess the role of loop>Y in BIG2 function, we compared the effect of BIG2/3A on TGN/RE architecture with the effects of BIG2 and BIG2/E738K. All BIG2 constructs were HA-tagged and expressed the correct molecular weight protein when transfected into HeLa cells (supplemental Fig. S1B).

Analysis of 13 cells expressing HA-BIG2 indicated that all contain the characteristic TGN marked by γ-AP1 (a representative cell is shown in Fig. 4). The exogenously expressed BIG2 co-localized with AP1. In contrast, cells expressing BIG2/E738K showed extensive tubulation of the AP1-containing
compartment as shown previously (26). Analysis of 18 transfected cells showed 12 (67%) containing TGN tubules. BIG2/E738K co-localized with AP1 in the tubules. Cells expressing BIG2/3A also showed extensive tubulation of the AP-containing elements, and BIG2/3A co-localized with AP1 in the tubules. The level of tubulation caused by BIG2/3A (13 cells of 16 showing tubulated TGN; i.e. 61%) is analogous to that caused by BIG2/E738K.

Similar results were obtained when RE architecture was probed by TfnR localization. Expression of BIG2 had a minimal effect on TfnR localization (supplemental Fig. S1D), whereas in cells expressing BIG2/E738K, TfnR was in tubulated structures that also contained BIG2/E738K. Expression of BIG2/3A also caused tubulation of TfnR-containing REs, and BIG2/3A co-localized with TfnR in the tubules. Thus, the BIG2/3A effect is analogous to the tubulation of TGN/RE caused by the inactive BIG2/E738K, suggesting that BIG2/3A might be catalytically compromised.

**Sec7 Domains with Mutations in Loop/H11022Do Not Catalyze GDP to GTP Exchange on ARF**—To probe directly the role of loop/H11022 in GDP to GTP exchange activity, we generated Sec7 domains from GBF1/7A (residues 690–894) and BIG2/3A (residues 645–832) and compared their catalytic activities with those of Sec7 domains of wild-type GBF1 and BIG2 and the

**FIGURE 3. Expression of GBF1/7A prevents de novo Golgi biogenesis.** HeLa cells were transfected with GFP-GBF1 (A), the catalytically inactive GFP-GBF1/E794K (B), or the GFP-GBF1/7A mutant (C). After 24 h, cells were either mock-treated (− panels), treated with BFA for 1 h (+ BFA panels), or treated with BFA followed by a 2-h washout (+ BFA + WO panels). Cells were processed for IF using anti-GFP and anti-GM130 antibodies. Insets show enlargements of boxed areas. Wild-type GBF1 supports Golgi reformation, whereas expression of GBF1/E794K and GBF1/7A prevents Golgi biogenesis.

**FIGURE 4. Expression of BIG2/3A causes tubulation of TGN.** HeLa cells transfected with HA-tagged wild-type BIG2, the catalytically inactive BIG2/E738K, or the BIG2/3A mutant were processed for IF using anti-HA and anti-γ-subunit of the AP1 adaptor complex antibodies. Transfected cells are marked with asterisks. Expression of BIG2/E738K and BIG2/3A causes membrane tubulation.
catalytically inactive GBF1/E794K and BIG2/E738K. We also tested whether loop J influences catalytic activity of ARNO Sec7d (residues 52–246). ARNO contains a phenylalanine at a position occupied by isoleucine in loop J of GBF1, BIG1, and BIG2 (Fig. 1B). Substituting Phe-243 with alanine generated ARNO/1A Sec7d, and its activity was compared with that of ARNO Sec7d and the inactive ARNO/E156K Sec7d.

GBF1, BIG2, and ARNO have been shown to catalyze GDP to GTP exchange on ARF1, and myristoylated ARF1 was used in the assay (27, 28). We were unable to measure exchange activity of wild-type and mutant GBF1 Sec7d (not shown) presumably because expressing GBF1 Sec7d in bacteria leads to incorrect folding and precipitation of GBF1 Sec7d in inclusion bodies (not shown). We readily detected exchange activity of BIG2 Sec7d and its activity was compared with that of ARNO Sec7d and the inactive ARNO/E156K Sec7d.

GBF1, BIG2, and ARNO have been shown to catalyze GDP to GTP exchange on ARF1, and myristoylated ARF1 was used in the assay (27, 28). We were unable to measure exchange activity of wild-type and mutant GBF1 Sec7d (not shown) presumably because expressing GBF1 Sec7d in bacteria leads to incorrect folding and precipitation of GBF1 Sec7d in inclusion bodies (not shown). We readily detected exchange activity of BIG2 Sec7d (Fig. 1B) as reported previously (25). In contrast, BIG2/E738K Sec7d lacked exchange activity. BIG2/3A Sec7d showed severely diminished exchange activity. GDP to GTP exchange was readily observed for ARNO Sec7d, whereas ARNO/E156K Sec7d showed significantly reduced catalytic activity (Fig. 5B). ARNO/1A Sec7d also showed decreased GDP to GTP exchange activity. Our results indicate that mutations within loop J of a large BFA-sensitive GEF (BIG2) and a small BFA-resistant GEF (ARNO) compromise GDP to GTP exchange activity of each GEF. A single F243A substitution in ARNO loop J inhibited GDP to GTP exchange to the same extent as the E156K mutation, naming this motif a critical determinant of exchange activity.

Mutations in Loop J Inhibit GDP to GTP Exchange via Distinct Mechanisms—The reduced GDP to GTP exchange by the Sec7d of BIG2/3A and ARNO/1A could be due to lack of ARF binding or the inability to catalyze GDP displacement. To provide a mechanistic understanding for BIG2/3A Sec7d and ARNO/1A Sec7d inactivity, we assessed their ability to bind ARF. We used a pulldown assay in which Sec7d was incubated with lysates prepared from HeLa cells expressing HA-tagged ARF1/T31N. ARF1/T31N is considered the GDP-arrested form of ARF1 and forms a stable complex with Sec7d (18, 21, 29). We readily detected ARF/T31N binding to beads containing wild-type BIG2 and ARNO Sec7d (Fig. 5C). An even higher amount of ARF1/T31N was recovered on beads containing the Sec7d from the catalytically inactive BIG2/E738K. This was expected based on the known stabilization of the ARF-Sec7d complex when GDP to GTP exchange is inhibited (21, 30). In contrast, ARF1/T31N was not recovered on beads containing the Sec7d from the BIG2/3A mutant. However, BIG2/3A appears to possess some ability to bind the ARF substrate as evidenced by the low level of exchange activity (Fig. 5A). Despite this, our data clearly document that the loop J region forms a critical interface for facilitating ARF docking.

As expected, ARF1/T31N was recovered on beads containing the Sec7d from wild-type ARNO and the catalytically inactive ARNO/E156K mutant (Fig. 5D). ARF1/T31N also bound to beads containing the Sec7d from the ARNO/1A mutant. Both ARNO/E156K and ARNO/1A appeared to bind less ARF1/T31N than wild-type ARNO. However, the slightly decreased level of substrate binding is unlikely to be the cause of the almost 50% decrease in catalytic activity (see Fig. 5B). Thus, it appears that ARNO/1A may have defects in both ARF binding and the ability to catalyze GDP to GTP exchange. These findings identify Phe-243 as a novel determinant of Sec7d function.

Bacterially produced wild-type GBF1 Sec7d did not bind ARF (not shown). Thus, we could not assess GBF1/7A binding.
to ARF in vitro. Instead, we used FRAP to assess ARF binding to full-length GFP-GBF1/7A in vivo. GFP-GBF1 has been shown to rapidly cycle on and off Golgi membranes with a $t_{1/2}$ of $\sim 16$ s, whereas the inactive GFP-GBF1/E794K that binds ARF but does not catalyze GDP to GTP exchange is stabilized on membranes with a FRAP of $t_{1/2} \sim 53$ s (18, 22, 29). Thus, if GFP-GBF1/7A binds ARF without catalyzing GDP to GTP exchange, its FRAP should be increased and similar to that of the inactive GBF1/E794K. We confirmed that wild-type GFP-GBF1 undergoes FRAP with a $t_{1/2}$ of $\sim 16$ s (supplemental Fig. S2). GFP-GBF1/7A undergoes FRAP with a $t_{1/2}$ of $\sim 16$ s within a morphologically normal Golgi. Similarly, GFP-GBF1/7A undergoes FRAP with a $t_{1/2}$ of $\sim 15$ s in cells in which GFP-GBF1/7A caused Golgi disruption and GBF1/7A localized in peripheral puncta. Thus, GFP-GBF1/7A cycles on and off membranes like wild-type GBF1 and significantly faster than the inactive GBF1/E794K. This suggests that GBF1/7A may be defective in ARF binding.

Our findings suggest that loop $\geq J$ is involved in both substrate binding and catalysis. Mutating seven or three residues in GBF1 loop $\geq J$ or three residues in BIG2 loop $\geq J$ appeared to compromise both ARF binding and GDP to GTP exchange. The single mutation of P243A in ARNO loop $\geq J$ allowed almost normal ARF binding but decreased GDP to GTP exchange. Thus, loop $\geq J$ is a novel determinant required for the exchange activity of multiple (if not all) Sec7d GEFs.

**DISCUSSION**

Crystal Structure of Human BIG2 Sec7d—Structures of Sec7d from yeast Gea1 (21) and Gea2 (32), mammalian ARNO and Grp1 (33), and RalF from Legionella pneumophila (34) are highly homologous. Our structure of BIG2 Sec7d closely parallels the previously determined molecular models, and superimposing BIG2 and Gea1 Sec7d showed close apposition of the main helical domains. Thus, BIG2 Sec7d is likely to use the same molecular mechanism of GDP displacement as other ARF GEFs. However, despite the overall similarity, there are many detailed differences in the positions of the helices and the connecting loops of BIG2 and Gea1. These differences may contribute to the selectivity of Sec7 domains for different ARFs.

Changes in sequence and structure of Sec7d from distinct GEFs may also reflect sites of regulatory inputs. For example, phosphorylation of Ser-883 in BIG1 in response to PKA activation decreases BIG1 activity (8). However, Ser-883 is not conserved in other GEFs. Thus, overall similarity in Sec7d structure might be essential for catalytic activity, whereas the subtle changes may fine tune the ARF specificity and/or the specificity of regulatory inputs for each GEF.

Sec7d Loop $\geq J$ Is a Novel Motif Required for ARF Activation—Multiple substitutions in loop $\geq J$ of GBF1 and BIG2 prevented ARF binding, identifying loop $\geq J$ as a key determinant in substrate interactions. The single amino acid substitution (P243A) in ARNO decreased ARF binding and inhibited GDP to GTP exchange. Thus, Phe-243 in ARNO may participate in both substrate binding and catalysis. Our data indicate that the previously unappreciated Phe-243 is as important as the glutamic finger Glu-156 for ARNO activity. Phe-243 in ARNO shows complete amino acid conservation in the mammalian members of the ARNO/Grp/cytohesin family and their paralogs in the fly, Xenopus, and zebrafish (33). It remains to be determined whether the analogous Ile-827 in BIG2 and Ile-885 in GBF1 also may participate in ARF binding and catalysis. Interestingly, Ile-827 of BIG2 and Ile-885 of GBF1 show complete amino acid conservation in human, fly, worm, yeast, and plant (6).

Our mutational analysis is consistent with the solved crystal structure and supports our conclusions that loop $\geq J$ directly participates in Sec7 function. However, alternative mechanisms may explain the observed inhibition in loop $\geq J$—compromised GEFs, and it remains possible that loop $\geq J$ acts by stabilizing the functionally relevant conformation of Sec7d without directly participating in substrate binding and catalysis.

Our studies also provide a molecular explanation for the finding that phosphorylation of Ser-883 within BIG1 decreases GEF activity (8). Ser-883 lies within the loop $\geq J$ region of BIG1 (KISM in BIG1; Fig. 1D) that is analogous to residues KIAM in BIG2 that we identified as essential for ARF binding. Thus, phosphorylation of Ser-883 is predicted to negatively affect ARF binding by introducing a strongly charged group into the ARF binding packet. The loop $\geq J$ of GBF1, BIG2, and ARNO does not contain a serine in an analogous position (Fig. 1B), making it unlikely that GBF1, BIG2, or ARNO is phosphorylated within loop $\geq J$. However, both GBF1 (35, 36) and BIG2 (8, 37) are phosphorylated, and it is possible that additional phosphorylation sites are present within the Sec7d to regulate their exchange activity.

The finding that loop $\geq J$ participates in GDP to GTP exchange and that it can be regulated by phosphorylation sets up an important paradigm in which GEF activity in generating activated ARF can be integrated with cellular homeostasis. For example, signals that activate PKA (PKA phosphorylates BIG1 Ser-883 (8)) will regulate both the physiological responses of a cell to specific stimuli and ARF activation that might be involved in the cellular response. This provides the means of linking extra- and intracellular signaling via PKC to ARF activity. ARFs are not known to be regulated through phosphorylation, and it is perhaps expected that the cell may phosphorylate the upstream GEFs to indirectly regulate ARF activity.

GEFs Compromised in ARF Binding Associate with Membranes and Act in Dominant Negative Manner in Cells—We and others have shown that GBF1 is stabilized on the membrane when bound to its ARF-GDP substrate (18, 29, 38), raising the possibility that membrane association of GBF1 (and perhaps BIG1/2) is mediated through binding to its ARF-GDP substrate. Previous work has shown that ARF1-GDP binds to the ER-Golgi SNARE membran, suggesting that a complex of membrane-associated ARF1-GDP might provide the binding site for GBF1 (31). Our BIG2/3A mutant showed greatly diminished ARF binding and provided a unique reagent to directly test the role of ARF-GDP in membrane association. We showed that BIG2/3A is efficiently targeted to membranes, indicating that efficient interactions with the ARF substrate are not required for membrane association. In addition, BIG2/3A is targeted correctly to the TGN/RE, suggesting that ARF substrates may play secondary roles in the selectivity of GEFs for a specific membrane.
C-terminal Regulatory Motif in Sec7 Domain

Catalytically inactive GBF1/E794K (9) and BIG2/E738K (26) cause dominantly negative effects in cells presumably by binding and sequestering ARF away from endogenous GEFs. This model predicts that GBF1/7A and BIG2/3A that were compromised in ARF binding will be silent when expressed in cells. However, both GBF1/7A and BIG2/3A acted as dominant negatives in cells. Thus, GBF1/7A and BIG2/3A appear to inhibit ARF activation in cells but by a mechanism that does not involve ARF sequestration. It is likely that GBF1/7A and BIG2/3A prevent ARF activation by competing with endogenous GEFs for membrane binding sites. This implies that endogenous GEFs activate ARFs only when both are associated with membranes.

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