GBF1: A Novel Golgi-associated BFA-resistant Guanine Nucleotide Exchange Factor That Displays Specificity for ADP-ribosylation Factor 5

Alejandro Claude,* Bao-Ping Zhao,* Craig E. Kuziemsky,* Sophie Dahan,‡ Scott J. Berger,§ Jian-Ping Yan,§ Adrian D. Armold,§ Eric M. Sullivan,§ and Paul Melançon*

*Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7; ‡Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada, H3A 2B2; and §Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309

Abstract. Expression cloning from a cDNA library prepared from a mutant CHO cell line with Golgi-specific resistance to Brefeldin A (BFA) identified a novel 206-kD protein with a Sec7 domain termed GBF1 for Golgi BFA resistance factor 1. Overexpression of GBF1 allowed transfected cells to maintain normal Golgi morphology and grow in the presence of BFA. Golgi-enriched membrane fractions from such transfected cells displayed normal levels of ADP ribosylation factors (ARFs) activation and coat protein recruitment that were, however, BFA resistant. Hexahistidine-tagged–GBF1 exhibited BFA-resistant guanine nucleotide exchange activity that appears specific towards ARF5 at physiological Mg^{2+}concentration. Characterization of cDNAs recovered from the mutant and wild-type parental lines established that transcripts in these cells had identical sequence and, therefore, that GBF1 was naturally BFA resistant. GBF1 was primarily cytosolic but a significant pool colocalized to a perinuclear structure with the β-subunit of COPI. Immunogold labeling showed highest density of GBF1 over Golgi cisternae and significant labeling over pleiomorphic smooth vesiculotubular structures. The BFA-resistant nature of GBF1 suggests involvement in retrograde traffic.

Key words: Golgi complex • Brefeldin A • Sec7 • ADP-ribosylation factor • protein traffic

BREFELDIN A (BFA)[1] is a small fungal heterocyclic lactone that blocks protein secretion (Misumi et al., 1986) and induces rapid and profound morphological changes in several organelles of the secretory pathway. In most cell types, BFA causes disassembly of the Golgi complex and redistribution of Golgi resident enzymes into the ER (Klausner et al., 1992; Pelham, 1991).

The earliest documented step in BFA action is inhibition of the recruitment of members of the ADP-ribosylation factor (ARF) family of GTPases onto membranes (Donaldson et al., 1992b; Helms and Rothman, 1992; Randazzo et al., 1993). The binding of ARFs to membranes is believed to provide high affinity binding sites for the Golgi coat protein COPI (Rothman and Orci, 1996). This preassembled complex of seven polypeptides is recruited to the membrane, initiating vesicle budding. In addition, the observation that ARFs stimulate phospholipase D activity on Golgi membranes (Brown et al., 1995; Ktistakis et al., 1996) suggests that ARFs may induce remodeling of the membranes by increasing the local levels of acidic phospholipids, which in turn may modulate recruitment of coat proteins (for review see Roth and Sternweis, 1997). Inhibition of ARF recruitment onto Golgi membranes, thus, prevents the assembly of the COPI complex and readily explains the effects of BFA on the secretory apparatus. The observation that BFA interferes with the recruitment of other proteins such as the Golgi-specific clathrin adaptors AP1 (Stamnes and Rothman, 1993; Traub et al., 1993) suggests the blocking of ARF-dependent recruitment of coat proteins as a general mechanism to explain the effects of BFA on all affected organelles.

BFA interferes with ARF recruitment by blocking the exchange of GTP for GDP, thereby preventing a conformational switch required for their association with Golgi membranes (Donaldson et al., 1992b; Helms and Rothman, 1992; Randazzo et al., 1993; Franco et al., 1995, 1996; Antonny et al., 1997; Losonczi and Prestegard, 1998). This exchange reaction is catalyzed by a membrane-associated

The current address of S. Dahan is Mayo Clinic, Rochester, MN 55905. The current address of J. Berger is Pacific Northwest National Lab, Richland, WA 99352. The current address of J.-P. Yan is Department of Biochemistry, Colorado State University, Fort Collins, CO 80523. Address correspondence to Paul Melançon, Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7. Tel.: (780) 492-6183. Fax: (780) 492-0450. E-mail: paul.melancon@ualberta.ca

1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; ARF-GEF, ARF-specific guanine nucleotide exchange activity; BFA, Brefeldin A; DPBS, Dulbecco's PBS; EBNA, Epstein-Barr nuclear antigen; G6PDH, glucose-6-phosphate dehydrogenase; GBF, Golgi-specific Brefeldin A resistance factor; KLH, keyhole limpet hemocyanin; LB, Luria-Bertani medium; NRK, normal rat kidney; PA, polyacrylamide; PNS, postnuclear supernatant.

guanine nucleotide exchange factor (GEF) that resists extraction with 1 M KCl and is protease sensitive (Donaldson et al., 1992b; Helms and Rothman, 1992; Randazzo et al., 1993). A BFA-sensitive exchange activity has also been detected in a large (>700 kD) soluble complex (Tsai et al., 1994; Morinaga et al., 1996), indicating that ARF-specific guanine nucleotide exchange activities (ARF-GEFs) may exist in dynamic equilibrium between membrane-bound and soluble pools.

We previously isolated several CHO mutant lines able to grow in the presence of BFA (Yan et al., 1994). Further characterization revealed that all of our mutant lines, termed BFY, had acquired Golgi-specific resistance. For example, whereas Golgi morphology and function became resistant to BFA in BFY cells, early endosomes retained their characteristic sensitivity (Yan et al., 1994). A similar study with an independently obtained collection of BFA-resistant CHO lines led to an identical conclusion (Torii et al., 1995). These results indicate that BFA acts by altering the function of related but distinct organelle specific targets that can be independently mutagenized and identified. In addition, they suggested that further analysis of BFY cells should identify a Golgi-specific BFA target.

Recently, a novel family of proteins implicated in ARF activation has been found to share the central domain of Sec7p (Sec7d). ARF-GEF activity was first reported for ARNO and Gea1p (Chardin et al., 1996; Peyroche et al., 1996). In addition, p200/ARF-GEP, a member of a large complex identified on the basis of its GEF activity, was subsequently shown to contain a Sec7d (Morinaga et al., 1996, 1997). The defining 170 residue Sec7d is highly conserved among the various members and was shown to have intrinsic ARF-GEF activity (Chardin et al., 1996; Sata et al., 1998; Mansour et al., 1999). Structural studies with the Sec7d of ARNO and GEA2p identified a critical glutamate residue that penetrates in the GTPase active site of ARFs to perturb Mg^{2+} binding and promote dissociation of the guanine nucleotide (Beraud-Dufour et al., 1998; Goldberg, 1998). This domain appears to be a direct target of BFA since the ARF-GEF activity of purified Sec7d from several proteins is sensitive to BFA (Sata et al., 1998; Mansour et al., 1999). Furthermore, mutations of key residues in this domain can dramatically alter its BFA sensitivity (Peyroche et al., 1999).

The various members of the Sec7d family likely act on different organelles and play distinct roles in protein traffic. To identify those members or their upstream regulators implicated in Golgi function, we used expression cloning to recover proteins relevant to growth inhibition by BFA. Here, we report the cloning and characterization of a novel Sec7d protein from a cDNA library prepared from one of our most resistant BFY lines. This 206-kD protein contains a Sec7 domain, localizes to the Golgi complex, and demonstrates BFA-resistant GEF activity that appears specific towards ARF5 at physiological Mg^{2+} concentration.

Materials and Methods

Reagents

ATP, UTP, creatine phosphate, and rabbit creatine phosphokinase were purchased from Boehringer Mannheim Biochemicals. Unless otherwise indicated, all other chemicals were obtained from Sigma Chemical Co. BFA was stored at −20ºC as a stock solution of 10 mg/ml (36 mM) in either 100% ethanol or DMSO. DNA and RNA purification kits and Ni-NTA resin were purchased from Qiagen. cDNA preparation kits were from GIBCO BRL. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, linker-adaptors, *Taq*, and Klenow DNA polymerases were from New England BioLabs Inc. The pCEP4, pRSETA, and pEBVHis-C plasmids and the TOP10 *Escherichia coli* strain were from Invitrogen Corp. Protein quantitation reagents and protein size markers were from Bio-Rad Laboratories. The radioactive nucleotides $\alpha[^{32}P]$GTP and $\gamma[^{35}S]$GTPγS were from NEN. Nitrocellulose membranes and filters were from MSI.

Tissue Culture

Media, culture reagents, Lipofectamine, and hygromycin B were purchased from Life Technologies Inc. Disposable plasticware and culture dishes were purchased from Falcon. The CHO^{pro-5} and 293-EBNA cell lines were purchased from American Type Culture Collection and Invitrogen Corp., respectively. The isolation of BFY-1 from the parental CHO^{pro-5} line was previously described (Yan et al., 1994). Normal rat kidney (NRK) cells were obtained from Dr. Thomas Hobman (University of Alberta, Edmonton, Alberta, Canada). The CHO^{pro-5} and BFY-1 mutant lines were maintained in suspension in α-MEM (GIBCO BRL) supplemented with 7.5% FCS (Sigma Chemical Co.), 100 μg/ml penicillin G, and 100 μg/ml streptomycin. Monolayers of 293-EBNA and NRK cells were maintained in DME supplemented with 10% FCS, 100 μg/ml penicillin G, and 100 μg/ml streptomycin.

Antibodies

The m3A5 mouse mAb that recognizes the 110-kD β-COP subunit and the antigiantin serum were supplied by the late Dr. Thomas Kreis (University of Geneva, Geneva, Switzerland) and Dr. E. Chan (Scripps Institute, La Jolla, CA), respectively. Rabbit anti–mouse IgG was obtained from Boehringer Mannheim Biochemicals. HRP-conjugated goat anti–mouse and anti–rabbit IgG were obtained from Bio-Rad Laboratories and Amersham Life Science, Inc., respectively. FITC-conjugated donkey anti–mouse and Texas red–conjugated donkey anti–rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. Goat anti–rabbit IgG-10 nm gold was from Sigma Chemical Co.

The peptide TDPIPTSEVN that corresponds to the carboxy-terminal sequence of Golgi-specific Brefeldin A resistance factor (GBF) 1 was synthesized by the Alberta Peptide Institute (University of Alberta) and cross-linked to keyhole limpet hemocyanin (KLH) or BSA. Female New Zealand rabbits were immunized using 200 μg of KLH-linked peptide emulsified 1:1 with Freund's complete adjuvant (Sigma Chemical Co.) and injected subcutaneously or intramuscularly in four sites (0.25 ml/site). Booster immunizations using 100 μg of KLH-linked peptide emulsified 1:1 with Freund's incomplete adjuvant were performed subcutaneously every 4 wk. Serum from rabbits H133, H134, and H154 displayed high titer and specificity by immunoblot analysis. Serum H133 was best for immunoelectron microscopy studies, whereas serum H154 was chosen for indirect immunofluorescence studies (Harlow and Lane, 1988).

Immunoblots

Immunoblots were carried out essentially as described (Harlow and Lane, 1988). For determination of β-COP levels, proteins transferred to nitrocellulose were probed with mAb m3A5 and detected by the enhanced chemiluminescence method using HRP-conjugated goat anti–mouse IgG. The results were quantitated using a scanner (Microtek E6). GBF1 levels were measured using the enhanced chemifluorescence method (Amersham) and a Storm (Molecular Dynamics, Inc.) fluorimetric scanner as per the manufacturer's instructions. Results were quantitated using the ImageQuant software program (Molecular Dynamics, Inc.).

Preparation of Subcellular Fractions

CHO cells grown in suspension were homogenized by repeated passage though the narrow bore of a ball homogenizer (Balch and Rothman, 1985). For preparation of homogenates, cells grown as monolayers were washed once in cold PBS before recovery by scraping into PBS containing 1 mM EDTA. Cells were washed once in buffer H (0.25 M sucrose/10 mM Tris, pH 8) and homogenized in the same buffer by 12 slow passages

through a 23-gauge needle. In all cases homogenization buffer was supplemented with 1 mM PMSF and the recommended concentrations of antipain, pepstatin, leupeptin, aprotinin, and E-64. Postnuclear supernatants were prepared by centrifugation of crude homogenates at 1,000 *g* for 10 min. Cytosols were prepared by desalting high speed (100,000 *g*) supernatants of crude homogenates over P6-DG (Bio-Rad Laboratories). Golgi-enriched membranes were obtained from crude homogenates by float-up on discontinuous sucrose density gradients as described (Balch et al., 1984).

To determine the distribution of GBF1, postnuclear supernatants (400 μg protein) were adjusted to 130 μl and spun at 100,000 *g* for 8 min in a Beckman TLA 100.2 rotor. After recovery of supernatants, pellets were washed with 3 ml buffer H and spun again. Washed microsomal pellets were resuspended in 120 μl buffer H containing 1% Triton X-100. Supernatants were adjusted to contain 1% Triton X-100 and 30 μl of each fraction were analyzed by immunoblots.

Detergent extracts were prepared either by incubating washed 150 cm^2 monolayers with 2.5 ml ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitors), or by resuspending washed cell pellets in 4 vol lysis buffer. After 5 min on ice, the lysed cells were passed 10 times through a 23-gauge needle and spun 10 min at 1,000 *g* at 4°C. Supernatants were stored at −70°C.

Purification of Native Bovine Brain ARFs

Partially purified ARFs were prepared from brain extracts using methods adapted from Taylor et al. (1992). In brief, bovine brain extracts were prepared from frozen brains (Pel-Freez; Rogers) and subjected to precipitation with ammonium sulfate. The protein pellet was resuspended in HKM buffer (25 mM Hepes, pH 7.1, 50 mM KCl, 1 mM $MgCl_2$) and desalted over a P6-DG column equilibrated in TM buffer (10 mM Tris, pH 7.9, 1 mM $MgCl_2$) containing 50 mM KCl. The eluate was adjusted with TM buffer to give a conductivity equivalent to 50 mM KCl before loading onto a Q-Sepharose (Pharmacia) column. The flowthrough from the Q-Sepharose column was adjusted with TM buffer to a conductivity equivalent to 20 mM KCl and run again over Q-Sepharose to allow ARF binding. ARFs were released with 50 mM KCl buffer and the eluate was concentrated by ultrafiltration over a YM10 membrane (Amicon). The concentrated sample was loaded onto a HiLoad 16/60 Superdex-75 column (Pharmacia) and ARF-containing fractions were eluted in 10 mM Tris, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$ and 10% glycerol. ARF fractions were identified using GTP–ligand blots (see above). The purity of pooled ARF fractions was estimated at 30% on the basis of its specific activity in GTP–ligand blots, using pure bovine ARF1 as standard. ARF1 and ARF3 were fully myristoylated and present at a ratio of 1:9, as determined by HPLC and mass spectrometry.

Purification of Recombinant Myristoylated ARF5

Cultures of BL21(DE3) harboring plasmids encoding human ARF5 (ARF5:PET21d+; Berger et al., 1998) and yeast *N*-myristoyl transferase (pBB131; Duronio et al., 1990) containing 100 μg/ml ampicillin and 20 μg/ml kanamycin were induced by addition of IPTG (0.5 mM) at an OD_{600} of 0.8. Bacteria were harvested by centrifugation after 4 h of growth at ambient temperature. The bacterial pellet was resuspended in 4 vol of lysis buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.02% Na^+azide) and supplemented with chicken egg lysozyme (1 mg/ml). After 30–60 min on ice, bacteria were passed three times through a French press in a chilled 1-inch cell (model FA-073; SLM/Aminco) and cellular debris were removed by centrifugation (10,000 *g*, 30 min, 4°C). ARFs were precipitated at 4°C by gradual addition of ammonium sulfate up to 40% saturation (at 0°C). Protein pellets were back extracted with 50 ml 10 mM Tris, pH 8, 1 mM $MgCl_2$ for 2 h. The solubilized protein fractions (~52 ml) were desalted on a Bio-Gel P-6 DG (Bio-Rad Laboratories) column (450 ml, 2.6 × 89 cm), equilibrated in desalting buffer (10 mM Tris, pH 8.0 at 4°C, 50 mM KCl, 1 mM $MgCl_2$) at 0.4 ml/min, and fractions containing protein were pooled for anion exchange chromatography. This desalted fraction was diluted with desalting buffer without KCl to a final conductivity at or below that of a 25-mM KCl standard, and then loaded onto a 175-ml Q-Sepharose fast flow column (FFQ; Pharmacia) equilibrated in desalting buffer with 25 mM KCl. ARF5 was eluted with a single-step gradient of 10 mM Tris, pH 8, 120 mM KCl, 1 mM $MgCl_2$.

The eluate was concentrated 10-fold over a YM10 membrane and the salt was adjusted to 60 mM using buffer exchange. The concentrated protein fraction was cleared by centrifugation before size exclusion chromatography on a Superdex 75 column (Pharmacia HiLoad 16/60 Prep Grade) equilibrated in desalting buffer with 10% glycerol. The major ARF-containing fractions were identified by SDS-PAGE and Coomassie staining, pooled, flash frozen, and stored at −70°C. HPLC analysis established that purity and extent of myristoylation were >90%.

Measurement of Membrane-associated Guanine Nucleotide Exchange Activity

ARF-GEF was measured using a filtration method adapted from the assay developed by Donaldson et al. (1992a). In brief, incubations were carried out in a 100-μl reaction mixture containing 25 mM Hepes, pH 8.0, 25 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10 μg BSA, 0.2 M sucrose, 1 μM $\alpha[^{32}P]$GTP ($1–2 \times 10^4$ cpm/pmol), 5.5 μg of semi-purified ARF proteins (~30% pure) and 4 μg of Golgi-enriched membranes in the presence or absence of BFA at 37°C for 1 h. The reaction was terminated by the addition of 2 ml cold 10 mM Hepes, pH 8.0, and the amount of protein-bound nucleotide was determined by filtration through nitrocellulose filters followed by five 2-ml washes with cold 10 mM Hepes, pH 8.0, solution. The extent of membrane-dependent nucleotide exchange occurring on ARFs specifically was calculated by correcting the signal from complete reactions, using background values measured in control incubations lacking ARFs or membranes. The membranes and ARF-independent nonspecific exchange corresponded to ~20–35% of the signal observed with complete reaction. BFA affected nonspecific nucleotide exchange by only ~5%, irrespective of which Golgi membranes were used. The Golgi extracts were normalized by protein concentration and UDP–galactosyl transferase activity (data not shown).

Measurement of Coatomer Recruitment

Assays were carried out as previously described (Yan et al., 1994). Each 200-μl coatomer binding assay contained 10 μg of Golgi-enriched membrane fractions prepared from BFA-sensitive 293 or from 293 cells overexpressing GBF1 and 90 μg of cytosol prepared from BFA-sensitive CHO^{pro-5} cells. After a 20-min incubation at 30°C in the presence of the indicated BFA concentrations, membranes were collected by centrifugation and resuspended in SDS sample buffer. The complete sample was loaded on 10% SDS gel and the relative amounts of membrane-associated β-COP were determined using immunoblotting as described above.

cDNA Library Preparation

Total RNA was extracted from 10^8 cells of the BFY-1 line and the parental line (CHO^{pro-5}) from which they were derived. Poly $(A)^+$ mRNA was purified over oligo-dT columns (Life Technologies) and used for cDNA synthesis according to the manufacturer's instructions (Superscript II; Life Technologies, Inc.). First-strand synthesis was primed using the provided oligo-dT/NotI primer-adapter, and a partially duplex HindIII primer with 5′ overhang (pAGCTCGAAGGGGTTCG; New England Biolabs) was blunt-end ligated to cDNAs after second-strand synthesis. Size analysis revealed cDNAs up to 20 kb in length, with the range of 0.5–11 kb well represented in both libraries. cDNAs were digested with NotI and ligated to the pCEP4 vector (digested with HindIII and NotI). The resulting libraries were transformed in *E. coli* TOP10 and displayed on plates. At least 2.5×10^5 colonies for each library were grown on Luria-Bertani medium (LB) plates, harvested, pooled, and stored in aliquots at −70°C.

Library Selection

Library aliquots were thawed and added to 1.5 liters of warm LB media and grown to a final OD_{600} of 0.4 (two to three generations). Plasmid DNA was isolated using a maxiprep kit (Qiagen). 293-EBNA cells were grown in T-175 flasks to ~60% density and transformed with 10 μg of the above libraries and 240 μg of Lipofectamine per flask, following the manufacturer's instructions. FCS was added after 6 h to 10% final concentration and the cells were further incubated for 18 h. The medium was replaced with complete DME containing 0.3 mg/ml hygromycin B and cells were cultured for 36 h to select transformants. At the end of this incubation, loose cells were removed and survivors were trypsinized and transferred to new flasks with complete DME containing 0.3 mg/ml hygromycin B. After 24 h, the hygromycin-sensitive cells that failed to reattach were removed and the surviving successful transformants (10–20% of initial population) were recovered by trypsinization. These cells were plated on Primaria dishes (Falcon) in complete DME with 0.3 mg/ml hygromycin B plus 0.4 μM BFA to select BFA-resistant transformants. The medium

was replaced every 24–36 h and BFA pressure was maintained until no survivors remained among control cells transformed in parallel with empty vector. At this point (usually 7–10 d), the BFA concentration was reduced to 0.2 μM and small colonies were allowed to grow for 2 wk. Surviving colonies were pooled and the plasmid DNA was recovered (Hirt, 1967). This DNA was electroporated in *E. coli*, the successful transformants were pooled, expanded in liquid culture, and plasmid DNA recovered by Midiprep (Qiagen). This enriched library was used in a new round of selection using the procedure described above. Since this expression system allows 50 episomes to be stably maintained per cell, the clone responsible for BFA resistance could be at best present at a frequency of 1 in 50 and could not be directly isolated by this methodology.

After two enrichment cycles, the recovered plasmids were electroporated in *E. coli*, and 100 colonies were selected at random and individually grown. Restriction analysis of miniprep DNA recovered from these clones showed that 33 contained inserts larger than 1 kb. Transformation of these plasmids into 293-EBNA cells, first in pools and then individually (diluted 50 times with empty vector), identified one plasmid, clone 32, that was able to confer BFA resistance. Dilution of clone 32 with empty pCEP4 was essential since transformation with pure clone 32 caused rapid death of the transformed cells. Dilution in the range between 5 to 50-fold appeared optimal since larger dilution caused a substantial decrease in the number of BFA-resistant colonies. This suggested that overexpression of the clone 32 gene product is toxic and, to survive, cells must adjust expression of this protein to provide BFA resistance while avoiding its toxic effects; this would be accomplished by altering the ratio of these two plasmids by asymmetric segregation during cell division. Dilutions lower than fivefold yielded fewer BFA-resistant transformants, presumably because increasing numbers of cells were transformed with levels of clone 32 that were immediately toxic. DNA sequencing was performed by primer walking on both strands using the ABI (Perkin-Elmer) sequencing kit. Ambiguities were resolved by standard dideoxy sequencing.

The transfections designed to test the enrichment of the BFA resistance factor during selection were carried out essentially as described above. Nearly confluent monolayers of 293 cells (2×10^7 cells) were transformed in triplicate and selection pressure was applied with 0.4 μM BFA for 1 wk and the surviving cells were transferred to new plates at various dilution ratios (1:2 to 1:100). After 1 wk of double selection in the presence of 0.3 mg/ml hygromycin B and 0.4 μM BFA, the hygromycin B concentration was gradually lowered by regularly exchanging half of the medium volume with fresh medium lacking the drug. BFA was maintained at 0.4 μM. The number of surviving colonies was quantitated 2 wk later and is presented ± SD.

Cloning of GBF1 from Wild-type cDNA Library

Approximately 5×10^5 clones from the CHO^{pro-5} cDNA library were displayed on LB plates and screened by colony hybridization using standard techniques (Ausubel, 1997). The α[^{32}P]dCTP labeled, random primed probe was generated using a Stratagene kit and the NheI-ScaI DNA fragment of GBF1, encompassing the first 2 kb at the 5′ end of the cDNA. Positive clones were characterized by Southern blotting (Ausubel, 1997) of restriction digests followed by standard dideoxy sequencing of both strands.

Production of $(His)_6$-tagged GBF1

A GBF1 coding fragment was excised from pCEP4 by digestion with NotI and SspI to yield a slightly truncated form of the gene missing the first five codons. This fragment was ligated to the vector pEBVHis-C (Invitrogen) that had been digested with XhoI (single polylinker site), filled in with Klenow polymerase to produce blunt ends, and followed by digestion with NotI. The resulting plasmid encodes an N-tagged $(His)_6$-GBF1 containing an additional 38 residues immediately preceding residue 6 of GBF1. Transformation of 293 cells with this construct yielded BFA-resistant cells at frequencies similar to those observed with the untagged version.

Detergent extracts were prepared as described above. Typically, 1–3 ml of extracts (5–15 mg of protein) were incubated overnight at 4°C with 0.05 ml of Ni-NTA resin (Qiagen) with gentle mixing. This slurry was packed on a mini column and 0.25 ml fractions were collected by gravity throughout the experiment. The column was first washed with 20-bed volumes of buffer N (50 mM $KHPO_4$, pH 8.0, 300 mM NaCl, 1 mM PMSF) containing 15 mM imidazole. Elution was performed with buffer N supplemented with 50 and 100 mM imidazole, using 20-bed volumes each time. The bulk of tagged GBF1 typically eluted with 50 mM imidazole.

Production of $(His)_6$-tagged Sec7 Domain of Sec7p

A DNA fragment containing the Sec7 domain encoding region (codons 827–1022) from the Sec7 gene of *Saccharomyces cerevisiae* was recovered by PCR using pTA33-1 (Achstetter et al., 1988) as a DNA template, Pfu DNA polymerase, and primers ySec7F 5′-CGTAGGATCCAGGAAAACTGCTTTATCGGAA-3′ and ySec7R 5′-CGTAGAATTCTCATTATGAAAGCATTGCCTGATGC-3′. After 12 reaction cycles (95°C/1 min, 48°C/50 s, 72°C/1 min 45 s) and a final extension period at 72°C for 4 min, the 0.6-kb fragment was digested with BamHI-EcoRI and subcloned into the prokaryotic expression vector pRSET-A (Invitrogen). The encoded product of 26 kD contained a hexahistidine sequence within the NH_2-terminal extension MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS. The construct was introduced into BL21DE3 pLYS-S and recombinant protein induced and purified as described (Mansour et al., 1999). Protein purity was unusually high (>90%) after Ni-NTA chromatography.

Measurement of GBF1-associated Guanine Nucleotide Exchange Activity on ARFs

The GTP exchange activity on ARF was measured as described (Paris et al., 1997). Reactions (100 μl) contained 50 mM Hepes, pH 7.5, 1 mM DTT, 1 mM or 1 μM of free Mg^{2+} (1 mM $MgCl_2$ and 2 mM EDTA), 1.5 mg/ml azolectin vesicles, 4 μM [^{35}S]GTPγS (6,000 cpm/pmol), and 1 μM ARF1/ARF3 (purified from bovine brain) or myristoylated recombinant ARF5. Reactions received either BFA from the 10 mM stock or an equal volume DMSO. Reactions were incubated at 37°C and unless otherwise indicated, aliquots (20 μl) were taken at 30 min. The results presented have been corrected by subtracting background values measured in absence of ARFs and/or GBF1. The assay of the Sec7d fragment of Sec7p was identical, except that it was carried out at 30°C and in the presence of myristoylated recombinant yeast ARF2 and 1 mM of free Mg^{2+}.

Cell Immunofluorescence

Wild-type CHO, BFY-1, and NRK cells grown to 60% confluency on fibronectin-coated glass coverslips were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilized for 5 min with 0.1% Triton X-100 + 0.05% SDS in PBS, and blocked with PBS + 0.2% gelatin. Cells were single- or double-stained by first incubating with optimal dilutions of m3A5 (anti–β-COP) and/or anti-GBF1 serum (H154) followed by Texas red–conjugated donkey anti–rabbit and/or FITC-conjugated donkey anti–mouse antibodies. 293 cells were processed identically, except that fixation was in 1:1 methanol/acetone for 5 min and incubated with antigiantin serum as the primary antibody. The coverslips were mounted using 80% glycerol in PBS and analyzed by standard epifluorescence using a Zeiss Axioscope microscope. Confocal analysis was performed on a Leica Aristoplan confocal laser scanning microscope (CLSM facility, University of Alberta). Images were processed for printing using Adobe Photoshop.

Immunoelectron Microscopy

Liver samples were prepared essentially as described previously (Dahan et al., 1994). In brief, livers obtained from overnight-fasted male Sprague-Dawley rats (100–125 g) were perfused first with saline and with a solution of 4% paraformaldehyde/0.5% glutaraldehyde/0.1 M phosphate buffer, pH 7.4, for 10 min. Small 1-mm^3 pieces were dissected out and left in the same fixative for another hour at 4°C. Liver samples were washed four times for 15 min with ice-cold 4% sucrose/0.1 M phosphate buffer, pH 7.4, cryoprotected with several changes of 2.3 M sucrose/0.1 M phosphate buffer (~1 h) (Tokuyasu, 1980), mounted on nickel stubs, and quick-frozen in liquid N_2. Cryosectioning of liver tissues samples was based on published procedures (Tokuyasu, 1980; Geuze et al., 1984; Griffiths et al., 1984) and was carried out as described in Dahan et al. (1994).

The immunolabeling procedure consisted of incubating the sections on drops of 0.02 M glycine in DPBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) for 10 min followed by incubation on primary antibody for 30 min at room temperature. Sections were washed 6×5 min in Dulbecco's PBS (DPBS) followed by blocking in DPBS-BCO (DPBS plus 2% BSA, 2% casein, and 0.5% ovalbumin) for 5 min and incubation in appropriate secondary antibodies conjugated to gold particles for 30 min. Sections were washed six times for 5 min in DPBS, six times for 5 min in H_2O, stained for 5 min with uranyl acetate–oxalate solution, pH 7.0, washed two times for 1.5 min in H_2O, and finally transferred to drops of methyl cellulose containing 0.4%

aqueous uranyl acetate for 10 min on ice. Grids were picked up with gold loops and excess methyl cellulose was removed with filter paper. Antibodies were diluted in DPBS-BCO as follows: 1:20 for mouse anti–β-COP, 1:5 for H133, 1:2 for H134, and 1:20 for all the secondary antibodies conjugated to colloidal gold. Controls where the primary antibodies were omitted revealed negligible labeling (not shown). Sections were viewed in a Philips 400 T electron microscope operating at 80 kV.

Quantitation of gold particle labeling was essentially as described in Dahan et al. (1994). Compartments of the secretory apparatus over which gold particles were scored are defined in the legend of Table I. The intertwining nature of vesicular/tubular profiles prevented direct measurement of membrane sectional profiles in tubule-rich areas. In this case, the surface of the entire tubular region was employed, including some cytoplasmic space, which may have led to a minor underestimation of gold labeling density.

Results

To identify proteins implicated in the mechanism of action of BFA, we used expression cloning to select cDNAs whose production confers growth advantage in the presence of BFA. We chose to clone from a library prepared from a highly BFA-resistant CHO cell line (BFY-1) to increase the chances of success since such a selection could yield either a mutant protein with altered BFA sensitivity or a wild-type protein whose overexpression overcame the effects of BFA. We expected to recover GBFs, since previous studies established that targets in this organelle were most critical to the effects of BFA on growth (Yan et al., 1994; Torii et al., 1995).

Identification of a cDNA Encoding a GBF

To recover GBF clones, we used an expression system based on the episomal Epstein-Barr virus (EBV)-derived vector, pCEP4, and its host cell line, 293-EBNA. This system allows high frequency isolation of stable transformants that maintain expression vectors as episomes that can subsequently be readily recovered for analysis. The high sensitivity of 293 cells to BFA ($LD_{50} < 0.07$ μM; Claude, A., and P. Melançon, unpublished observation) makes them particularly appropriate hosts for the selection procedure.

Transformation of 293 cells under standardized conditions with the BFY-1 cDNA library yielded stable transformants able to grow in the presence of 0.2 μM BFA at a frequency of $\sim 10 \pm 3$ per 10^6 hygromycin-resistant transformants, significantly higher than observed in parallel transfections with empty vector. Transformation of pooled plasmids recovered from BFA-resistant colonies grown to confluence (enriched libraries) yielded BFA-resistant transformants at a frequency of $1.4 \times 10^3 \pm 0.2$ per 10^6 hygromycin-resistant transformants, indicating a greater than 100-fold enrichment in plasmids able to promote BFA resistance (see Materials and Methods for details). These results confirmed the presence of cDNAs encoding GBFs in the BFY-1 library and demonstrated the effectiveness of our selection method. Transformation with a twice-selected library yielded a very large number of BFA-resistant transformants (Fig. 1). Screening of 100 plasmids recovered from this library, first in pools then singly, yielded clone 32. Its insert of 6.8-kb insert was designated GBF1 (Fig. 1; see Materials and Methods for details).

To determine whether the cDNA insert in clone 32, renamed pCEP4-GBF1, is the most abundant or likely candidate for encoding a BFA resistance factor, several independent BFA-resistant 293 colonies were isolated after transformation with an enriched BFY-1 library. Four such successfully expanded colonies yielded plasmid preparations that conferred BFA resistance; further analysis by restriction mapping, Southern blots, and colony hybridization demonstrated that all four plasmid preparations were similarly enriched ($\sim$1 in 45) in plasmids containing inserts that hybridized with and had the same size and restriction pattern as GBF1 cDNA (not shown). Furthermore, colony hybridization of several independently enriched BFY-1 libraries demonstrated enrichment of the same cDNA to frequencies also in the range of 1:30 to 1:60, the maximum expected with this approach. These observations did not result from a high abundance in GBF1 mRNA since Southern and Northern blot analysis indicated that it was relatively rare and present at $<$1:100,000 copies in the original libraries (not shown). We conclude that GBF1 is the most likely candidate for a BFA resistance factor encoded in the BFY-1 cDNA library.

GBF Is a Novel 206-kD Protein Containing a Sec7 Domain

Analysis of the predicted amino acid sequence (Fig. 2) revealed a novel 206-kD protein with significant homology to a family of proteins that contains a 170-residue domain, called Sec7 domain (Sec7d), first identified in the secretion protein Sec7p of *S. cerevisiae*. This homology is significant since many Sec7 domain–containing proteins have been implicated in ARF guanine nucleotide exchange (Chardin et al., 1996; Peyroche et al., 1996; Meacci et al., 1997). Furthermore, the Sec7d itself has been shown to possess an intrinsic ARF-GEF activity (Chardin et al., 1996; Morinaga et al., 1997; Sata et al., 1998; Mansour et al., 1999).

The Sec7d of GBF1, highlighted in Fig. 2 A, displays an identity ranging from 38 to 45% with that of the other members and carries the two canonical motifs (boxed). More importantly, it contains the conserved glutamate of motif 1 (FRLPGEAPVI) recently implicated in the GEF activity of ARNO (Beraud-Dufour et al., 1998; Goldberg, 1998). Multiple alignment of GBF1 with key members of this family showed most extensive similarity with the two yeast proteins, Gea1p and Gea2p (Fig. 2 B). In addition to the defining central Sec7d, these proteins share eight regions ranging from 23–38% and 44–64% in extent of identity and similarity, respectively. In contrast, p200 and Sec7p share only five and three of these regions with GBF1, respectively, and, thus, appear to fall in a separate class. Small proteins of this family, such as ARNO only share the Sec7d. Further analysis of GBF1 with computer prediction programs did not reveal additional salient features other than a hydrophobic segment between residues 1,633–1,651 and a proline-rich region at the COOH terminus starting at residue 1,778.

Overexpression of GBF1 Confers BFA Resistance Both In Vivo and In Vitro

To determine if the growth advantage resulting from GBF1 expression correlated with stabilization of the Golgi

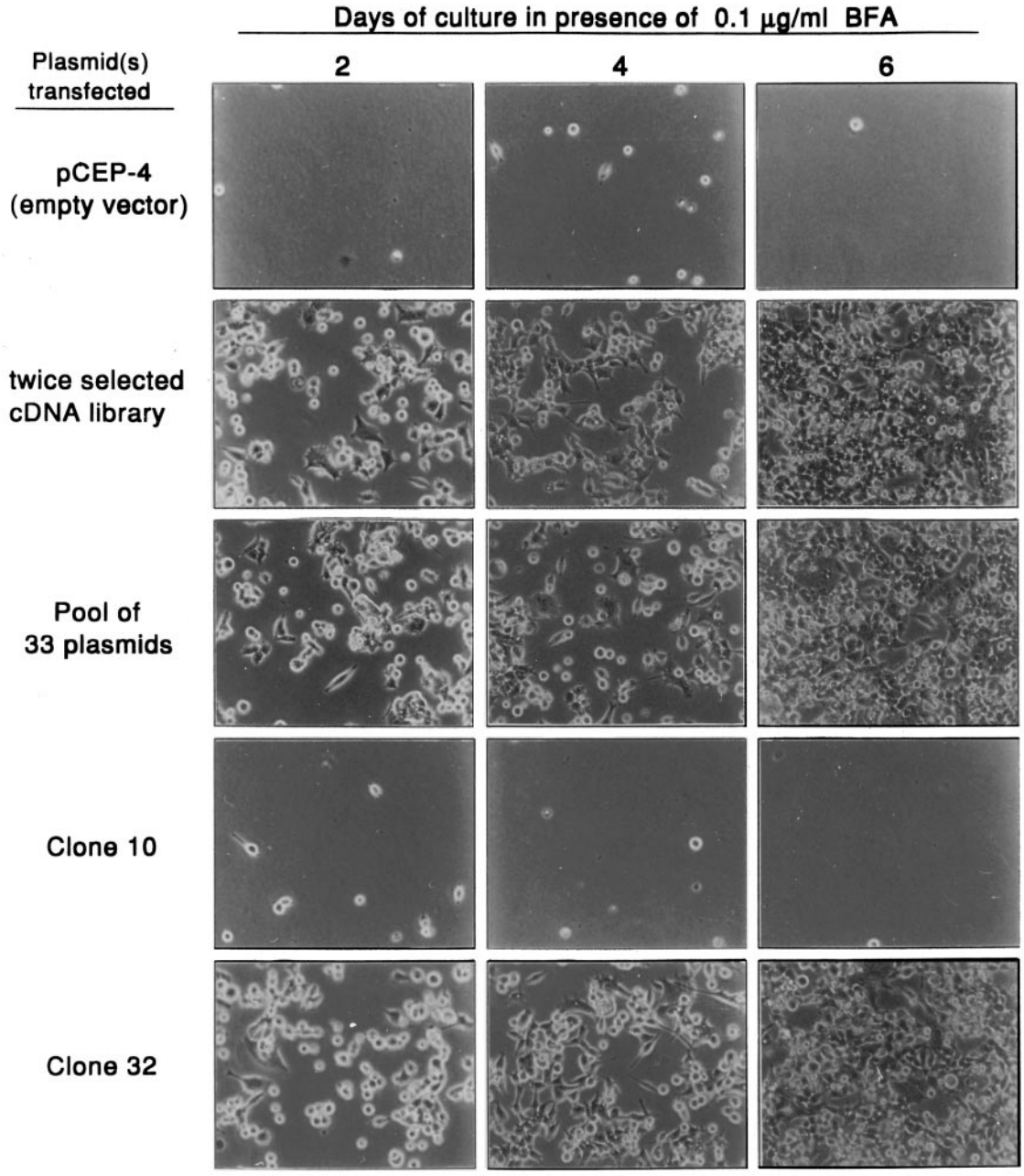

Figure 1. Isolation of a cDNA clone that allows cell growth in the presence of BFA. 293 cells were transformed as described in Materials and Methods with pCEP4 (empty vector), a twice-enriched BFY1 cDNA library, a pool of 33 plasmids derived from the twice-enriched cDNA library, clone 10 and clone 32 (both included in the pool of 33 plasmids). Transformants were selected and grown in the presence of 0.4 μM (0.1 μg/ml) BFA for 6 d. Cell growth was scored by light microscopy and photographs were taken at the indicated intervals.

complex, we tested the effect of BFA on the distribution of Golgi markers in control and GBF1-expressing cells. Such stable GBF1 transformants grew at BFA concentrations 10–15-fold higher than control transformants containing empty vector and maintained this level of resistance for at least 4 mo of continuous culture. Giantin, a well characterized Golgi complex marker (Linstedt and Hauri, 1993) was used to evaluate the morphology of GBF1 transformed cells. As shown in Fig. 3, expression of GBF1 allowed cells to maintain the characteristic perinuclear localization of their Golgi complex in the presence of 4 μM BFA, a concentration that led to complete dispersal in control cells.

It has been previously reported that BFA inhibits ARF activation and coat recruitment both in vivo and in vitro, indicating that BFA acts at or upstream of the ARF guanine nucleotide exchange activity (Donaldson et al., 1992b; Helms and Rothman, 1992; Randazzo et al., 1993). To test whether GBF1 acts at this level, we used cell-free assays that measure membrane-associated ARF-GEF ac-

A

MVDKNIYIIQGEINIVVGAIKRNARWSTHIPLDEERDPLLHSFSHLKEVLNSVTE
LSEIEPNVFLRPFLEVIRSEDTTGPITGLALTSVNKFLSYALIDPTHEGTAEGME
NMADAVTHARFVGTDPASDEVVLMKILQVLRTLLLTPVGTHLTNESVCEIMQSCF
RICFEMRLSELLRKSAEHTLVDMVQLLFTRLPQFKEEPKSYVGTNMKKLKMRAGG
MSDSSKWKKQKRSPRPPRHMTRVTPGSELPAPNGATLSCNLTSGMPFIDVPSSIS
SASSEAASAVVSPCTDSGLELSSQTTSKEDLTDLEQAGSPRESTTTESGSNEIGV
SDQLDPQEGSHVEKAQSASVESIPEVLEECTSPPDHSASVHDMDYVNPRGVRFTQ
SSQKEGTALVPYGLPCIRELFRFLISLTNPHDRHNSEGMIHMGLHLLTVALESAP
VAQCQTLLGLIKDEMCRHLFQLLSVERLNLYAASLRVCFLLFESMREHLKFQLEM
YMKKLMEIITVENPKMPYEMKEMALEAIVQLWRIPSFVTELYINYDCDYYCANLF
EDLTKLLSKNAFPVSGQLYTTHLLSLDALLTVIDSTEAHCQAKVLNTLTQQEKKE
TSRPSYEAVDSTQEANSTERATIDGKATGMASDALGLHLQSGGWLSAEHGKPRCN
DVEEAGDSGADKKFTRKPPRFSCLLPDPRELIEIKNKKKLLITGTEQ**FNQKPKKG**
IQFLQEKGLLTIPMDNTEVAQWLRENPRLDKKMIGEFVSDRKNIDLLESFVSTFS
FQGLRLDEALRLYLEAFRLPGEAPVIHRLLEAFTEHWRSCNGSPFANSDACFALA
YAVIMLNTDQHNHNVRKQNVPMTLEEFRKNLKGVNGGKDFEQDILEDMYHAIKNE
EIVMPEEQTGLVRENYVWSVLLHRGATPEGIFLRVPPGSYDLDLFTMTWGPTIAA
LSYVFDKSIEETIIQKAISGFRKCAMISAHYGLSDVFDNLIISLCKFTALSSESI
ENLPTVFGSNPKAHIAAKTVFHLAHRHGDILREGWKNIMEAVLQLFRAQLLPQAM
VEVEDFVDPNGKISLQREEMPSNRGESSVLSFVSWLTLSGPEQSSVRGPSTENQE
AKRVALDCIKQCDPEKMITESKFLQLESLQELMKALVSVTADEETYDEEDAAFCL
EMLLRIVLENRDRVGCVWQTVRDHLYHLCVQAQDFCFLVERAVVGLLRLAIRLLR
REEISGQVLLSLRILLLMKPSVLSRVSHQVAYGLHELLKTNAANIHSGDDWATLF
TLLECIGSGVKPPDALQATARADAPDAGAQSDSELPSYHQNDVSLDRGYTSDSEV
YTDHGRPGKIHRSATDADMVNSGWLVVGKDDIDNSKAGAGLSRPSPSPLVNQYSL
TVGLDLGPHDTKSLLKCVESLSFIVRDAAHITPDNFELCVKTLRIFVEASLNGGC
KSQDKRGKSHKYDSKGNRFKKKPKEGSVLRRPRTSSQHGTRGGHSDEEEDEGVPA
SYHTVSLQVSQDLLDLMHTLHTRAASIYSSWAEEQRHLESGGRKIEADSRTLWAH
CWCPLLQGIACLCCDARRQVRMQALTYLQRALLVHDLQKLDALEWESCFNKVLFP
LLTKLLENISPADVGGMEETRMRASTLLSKVFLQHLSPLLSLSTFAALWLTILDF
MDKYMHAGSSDLLSEAIPESLKNMLLVMDTAEIFHSADARGGSPSALWEITWERI
DCFLPHLRDELFKQTVIQDPMPTEPHSQNALASTHLTPAAGDPGHLPSPEIPSEV
GACDSEKPEGTRATSSSSPGSPVASSPSRLSPSPEGPPPLAQPPLILQPLTSPLQ
VGVPPMALPIILNPALIEATSPVPLLSTPRPTDPIPTSEVN

B

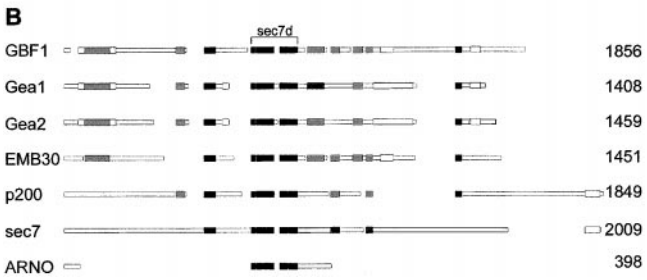

Figure 2. GBF1 is a novel member of the Sec7 family of proteins. (A) Deduced amino acid sequence of GBF1. The Sec7 domain of this protein (170 amino acids) is indicated in bold characters. The two highly conserved motif 1 and motif 2 within this domain are surrounded by boxes. The sequence data are available from GenBank/EMBL/DDBJ under accession number AF127523. (B) Multiple alignment of GBF1 and other key members of the Sec7 family of proteins generated with the MACAW program. The name of each protein is indicated on the left of each peptide diagram. The number of amino acid residues is indicated on the right. Boxes indicate regions of significant homology among two or more proteins. Darker shading of homology boxes indicates increased representation of these domains across family members. Small members of the family are represented by the better characterized ARNO.

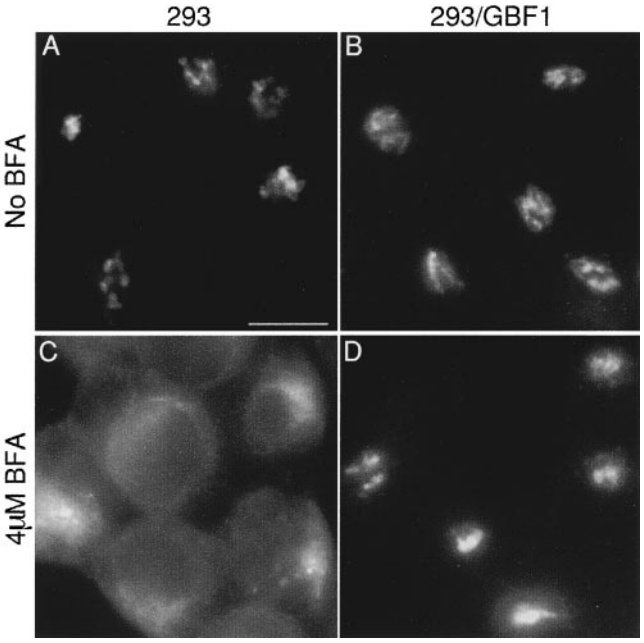

Figure 3. The Golgi morphology of 293 overexpressing GBF1 is resistant to BFA. 293 cells were transformed as described with pCEP4 vector (control) or pCEP4-GBF1 (GBF1) and grown on coverslips. The cells were incubated in the absence (A and B) or in the presence of 4 μM BFA (C and D) for 20 min before fixation and staining with antigiantin serum as described in Materials and Methods. Bar, 10 μm.

tivity and recruitment of COPI. Golgi-enriched membrane fractions were prepared from either control or GBF1-expressing 293 cells. GEF assays performed with native ARFs obtained from bovine brain (predominantly ARF3) established that Golgi-enriched membrane fractions prepared from 293/GBF1 cells displayed normal levels of ARF-GEF activity (Fig. 4 A). However, in contrast to that observed with control membranes, this activity was completely resistant to BFA. To establish whether the BFA-resistant ARF-GEF activity was relevant to coatomer recruitment, we compared the ability of 293 and 293/GBF1 membranes to recruit COPI components. As observed with the nucleotide exchange assay, 293/GBF1 membranes recruited levels of COPI nearly identical to those measured with control membranes (Fig. 4 B). Furthermore, COPI recruitment on these membranes was barely affected at a BFA concentration as high as 70 μM when recruitment to control membranes was inhibited by >50% at 7 μM BFA (Fig. 4 B).

To measure the extent of GBF1 overexpression and assess its distribution, we prepared and characterized several antisera raised against a peptide corresponding to the COOH terminus of GBF1 (see Materials and Methods). These antisera recognized specifically a protein of 206 kD in both CHO and 293 cells, a size similar to that predicted from the sequence of the cDNA (Fig. 5 A and data not shown). BFA-resistant 293 transformants overexpressed GBF1 six- to eightfold above the endogenous protein level (Fig. 5 A, right). As predicted, the majority of overexpressed GBF1 in 293/GBF1 cells was recovered in cytosolic extracts under conditions where microsomes were efficiently removed as established with the membrane protein calnexin (Fig. 5 B). Endogenous GBF1 in BFY-1 cells also partitioned primarily to the cytosol (Fig. 5 C); quantitation of this and similar experiments established that only a small fraction (< 10%) of endogenous protein was recovered in the microsome pellet. Similar results were obtained for the endogenous protein in wild-type 293 and

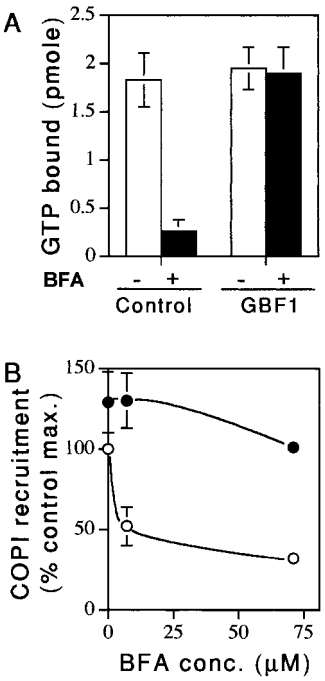

Figure 4. ARF-GEF and COPI recruitment activity on Golgi membranes from cells expressing GBF1 is resistant to BFA. (A) Identical amounts (4.2 μg) of Golgi-enriched membrane fractions from 293 cells or 293 cells transformed with GBF1 were assayed for ARF nucleotide exchange in the presence of α[^{32}P]GTP and in the absence or presence of BFA (280 μM). Each bar represents the average of three determinations ± SD. Similar results were obtained with several independently obtained membrane preparations. (B) Coatomer recruitment assays contained Golgi membranes prepared from 293 cells transformed with either pCEP4 (open circle) or pCEP4-GBF1 (closed circle) and the indicated amounts of BFA. The extent of recruitment of COPI provided by BFA-sensitive cytosol was determined in triplicate as described in Materials and Methods. The results ± SD are plotted as a function of BFA concentration. Similar results were obtained with several different membrane preparations.

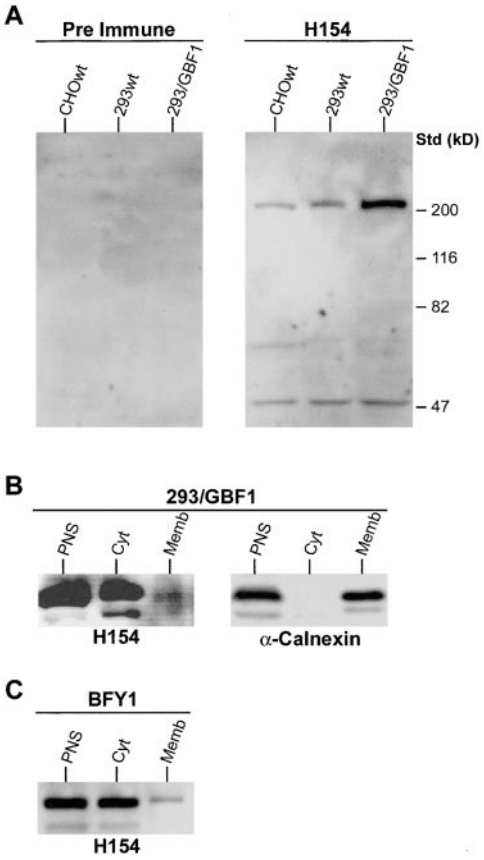

Figure 5. The anti–C-tail peptide antibody detects a 206-kD protein consistent with the predicted size of GBF1. (A) Serum H154 recognizes a 206-kD protein that is expressed at normal levels in mutant BFY-1 cells but is overexpressed in 293 cells transfected with pCEP4-GBF1. Identical amounts of Triton X-100 extracts (30 μg) from the indicated cell lines were analyzed by PAGE/immunoblotting as described in Materials and Methods. Blots were incubated with the indicated sera and processed for enhanced chemifluorescence. Scans obtained with a PhosphorImager in fluorescence mode are shown. The size of molecular weight standards run in parallel are indicated. (B) GBF1 is primarily cytosolic when overexpressed in 293/GBF1 cells. Identical amount (90 μg) of postnuclear supernatants from the indicated cells were analyzed by PAGE/immunoblotting either directly or after separation into cytosol and membrane fractions by ultracentrifugation as described in Materials and Methods. Blots were incubated with H154 anti-GBF1 serum and anticalnexin antibodies before processing for enhanced chemifluorescence. The distribution of the membrane protein calnexin confirms the absence of microsomes in the cytosol and their recovery in the pellet fraction. (C) GBF1 is primarily cytosolic in BFY-1 cells. The fractionation and detection were as described for B.

CHO cells (not shown). Our observations suggest that GBF1 is a primarily soluble protein implicated in coatomer recruitment.

GBF1 Is a BFA-resistant ARF-GEF

To confirm that GBF1 had ARF-GEF activity and determine whether this activity was sensitive to BFA, we modified GBF1 with a hexahistidine tag to facilitate its purification. Control transfection experiments established that tagging did not reduce the ability of GBF1 to cause BFA resistance (not shown). A significant fraction of GBF1 from detergent extracts of $(His)_6$-GBF1 transformants bound Ni-NTA columns and eluted at a 50 mM imidazole concentration (not shown). In contrast, endogenous GBF1 in extracts from control cells remained in the flowthrough fraction. At 1 μM of free Mg^{2+}, eluate fractions containing tagged-GBF1 stimulated binding of GTP on native ARFs from the bovine brain (1:9 mixture of ARF1 and ARF3), whereas those from control cells showed no activity (Fig. 6 A). This GEF activity appears specific for small GTPases of the ARF family since no such stimulation was observed with purified Sar1p or rab1b (Fig. 6 A). The GEF activity observed on ARF1/3 is significant and clearly above the spontaneous loading observed with ARF alone. This background was much lower than previously reported by Paris et al. (1997) for recombinant myristoylated ARF1 that indicates that ARF3 spontaneously binds GTP at a much lower rate than ARF1 at low Mg^{2+} concentrations.

As predicted from the studies with Golgi-enriched fractions in Fig. 4, the GEF activity of GBF1 towards ARF1/3 is completely resistant to BFA under these conditions. Addition of 360 μM BFA to exchange assays caused no reduction in nucleotide exchange (Fig. 6 B). As a positive control to establish the activity of our BFA in in vitro exchange assays, we constructed, purified, and tested a 36-kD recombinant protein containing the Sec7 domain of Sec7p. Such a truncated protein was previously shown to have BFA-sensitive ARF-GEF activity (Sata et al., 1998). BFA caused dose-dependent inhibition of our recombinant protein (Fig. 6 C), therefore, confirming the BFA-resistant nature of GBF1 observed in Fig. 6 B.

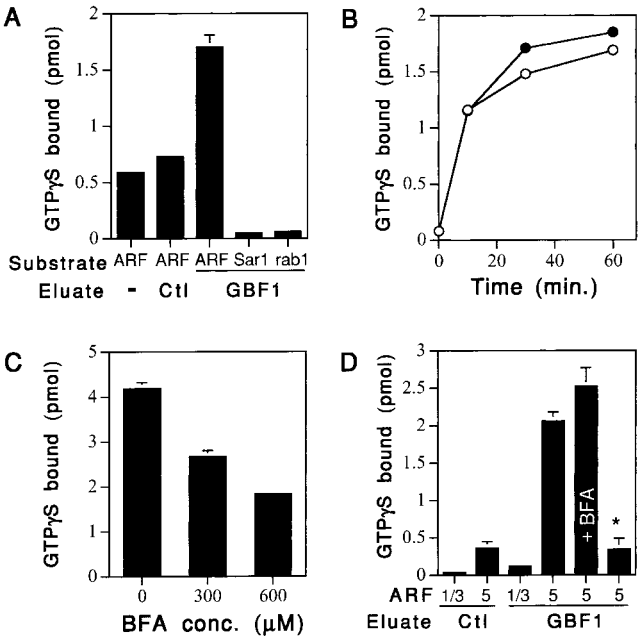

Figure 6. $(His)_6$-GBF1 is a BFA-resistant ARF-GEF. (A) Fractions enriched in $(His)_6$-GBF1 display a GEF specific for ARFs. Identical volumes (5 μl) of the 50 mM imidazole eluate fractions from the control (vector alone) and $(His)_6$GBF1 extracts were assayed for loading of small GTPases with GTPγS in 1 μM of free Mg^{2+} as described in Materials and Methods. Purified ARF1/3, Sar1p, or rab1b (1 μM each) were used as substrate as indicated. Each bar represents the average of three determinations ± SD. (B) GEF activity of GBF1 towards ARF1/3 is BFA resistant. Time course of ARF1/3 nucleotide exchange using the 50 mM imidazole eluate fraction from the $(His)_6$GBF1 extracts. Assays were performed at 1 μM of free Mg^{2+} either in the presence (closed circle) or absence (open circle) of BFA (360 μM). Similar results were obtained with several independent preparations. (C) The purified Sec7 domain of Sec7 is a BFA-sensitive ARF-GEF. Assays were performed as described in Materials and Methods with the indicated amounts of BFA. Each bar represents the average of three determinations ± SD. (D) GBF1 is a BFA-resistant GEF specific for ARF5 at 1 mM Mg^{2+}. Identical volumes (5 μl) of the 50 mM imidazole eluate fractions from the control (empty vector) and $(His)_6$GBF1 extracts were assayed for loading of ARF1/3 or ARF5 in assays containing 1 mM Mg^{2+}. BFA was used at a concentration of 600 μM. One set of reactions was performed with the GBF1 fraction inactivated (asterisk) by incubation at 95°C for 5 min. Each bar represents the average of three determinations ± SD.

The initial characterization of GBF1 was performed at low Mg^{2+}concentrations because its GEF activity towards ARF1/3 appeared to be very poor at the higher and more physiological Mg^{2+}concentration of 1 mM. Further analysis revealed that this resulted not from an overall weak activity but rather from its specificity towards group II ARFs. As shown, in Fig. 6 D, GBF1 effectively promoted GTP loading on ARF5 but remained inactive on ARF1/3 at physiological Mg^{2+} concentration. Importantly, this GEF activity of GBF1 was fully resistant to 600 μM BFA. Consistent with the proposed ARF5-GEF activity of GBF1, neither control extracts nor heat-treated GBF1 fractions (Fig. 6 D, asterisk) stimulated GTP binding. Note that although moderate, the extent of ARF loading observed here was comparable to that previously reported for other large ARF-GEFs (Peyroche et al., 1996; Morinaga et al., 1997; Mansour et al., 1999).

Table I. Expression of GBF1 cDNAs Recovered from Both BFY-1 and Wild-type CHO Cells Confers Resistance to BFA

Plasmid transfected	Percent BFA-resistant cells*
pCEP4	3 ± 1.2
pCEP4-GBF1	60 ± 2
pCEP4-$GBF1_{WT}$	64 ± 12

*293 cells were transformed with empty vector (pCEP4), the plasmid first recovered from the BFY-1 cDNA library (pCEP4-GBF1) and a plasmid recovered from the wild-type cDNA library illustrated in Fig. 7 A (pCEP4-$GBF1_{WT}$). An equal number of hygromycin B–resistant cells were plated in the presence of 0.4 μM BFA and incubated for 3 d. Surviving cells were trypsinized and counted. The results ± SD are presented as a percentage of hygromycin B–resistant transformants that are BFA resistant.

Wild-type Form of GBF1 Is a Naturally Resistant ARF-GEF

To determine whether GBF1 recovered from the BFY-1 library was a mutant allele, we isolated cDNAs from a library prepared from the wild-type parental CHO line by colony hybridization. Sequencing revealed that full length cDNAs recovered from the wild-type library had sequences identical to GBF1. Deletions of 3 and 12 nucleotides were observed at positions 1,864 and 4,479, respectively (Fig. 7 A), and reverse transcriptase–PCR analysis of mRNA prepared from both BFY-1 and parental CHO cells established that transcripts with and without those deletions were present at identical frequencies in both mutant and wild-type lines (not shown). Those probably arose by alternative processing. Furthermore, cDNAs identical in sequence to that shown in Fig. 7 A were recovered by colony hybridization of a twice selected BFY-1 cDNA library. The fact that GBF1 transcripts with identical sequences were recovered from wild-type and BFY-1 cells indicate that wild-type GBF1 is naturally BFA resistant. As expected, transfection of the wild-type cDNA diagrammed in Fig. 7 A led to recovery of BFA-resistant transformants (Table I). The human orthologue of GBF1 (Mansour et al., 1998) similarly caused BFA resistance when overexpressed (Melançon, P., unpublished observations).

The previous results established that BFA resistance in BFY-1 cells did not arise by mutation of GBF1. To test whether BFY-1 became resistant to BFA by overexpressing this BFA-resistant GEF, several independent Triton X-100 extracts were obtained from the wild-type parental and BFY-1 cell lines and analyzed for GBF1 content by immunoblots. Lanes loaded with equal amounts of protein, as confirmed with the cytosolic marker glucose-6-phosphate dehydrogenase (G6PDH), contained nearly identical levels of GBF1 (Fig. 7 B). Multiple quantitation of several independent extracts from these lines showed that endogenous GBF1 levels in those lines were within 10% of each other. This observation ruled out overexpression of GBF1 as the mechanism of resistance in BFY-1 cells.

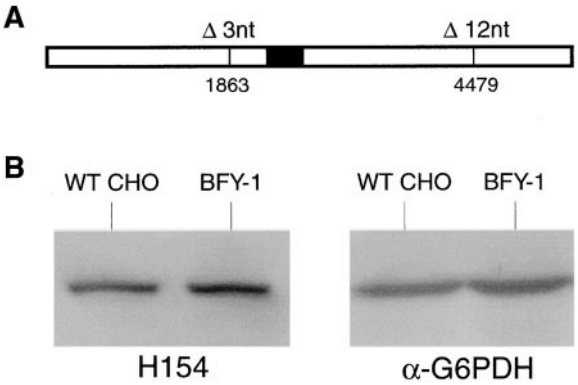

Figure 7. GBF1 is a naturally BFA-resistant GEF that is not overexpressed in BFY-1 cells. (A) Schematic representation of the GBF1 allele identified in wild-type CHO cells. The position of the Sec7d is indicated by the dark rectangle and the deletions by thin vertical lines. The size of the deletions and the position of the last nucleotide before each deletion are indicated above and below, respectively. These in frame deletions lead to the loss of S (622) and VSQD (1494-1497), respectively. (B) GBF1 is expressed at similar levels in wild-type CHO and mutant BFY-1 cell lines. Identical amounts of Triton X-100 extracts (30 μg) from the indicated cell lines were analyzed by PAGE/immunoblotting as described in Materials and Methods. Blots were incubated with H154 anti-GBF1 and anti-G6PDH antibodies. The ratios of GBF1 to G6PDH were identical in both extracts.

GBF1 Colocalizes with β-COP to the Golgi Complex

The observation that GBF1 is a BFA-resistant ARF-GEF whose expression allows BFA-resistant recruitment of the COPI coat onto Golgi membranes strongly implicates it in protein traffic at the Golgi complex. To determine if GBF1 associates with the Golgi complex in vivo, we examined its intracellular distribution in CHO and NRK cells using indirect immunofluorescence with the H154 antiserum (Fig. 8).

The images shown in Fig. 8 A revealed significant cytosolic staining accompanied with clear localization to a perinuclear structure reminiscent of the Golgi complex. As expected from the similar levels of expression and distribution between membrane and cytosol fractions of wild-type CHO and BFY-1 cells, comparable distribution was observed in these two cell lines. In the flatter NRK cells with better morphology, GBF1 stained a tight ribbonlike perinuclear structure, characteristic of the Golgi complex in these cells (Fig. 8 A, right). To confirm the link between GBF1 and COPI, we examined by confocal microscopy the intracellular distribution of these two proteins in NRK cells stained simultaneously with H154 and m3A5, a well characterized antibody that recognizes the β-subunit of COPI (Fig. 8 B). The extensive overlap in the distribution of endogenous GBF1 and β-COP shown by the merged image in the center confirms that the primary site of membrane recruitment in the cell is the Golgi complex.

More detailed subcellular localization of GBF1 was obtained by immunoelectron microscopy. Immunolabeling of liver ultrathin cryosections was performed with several sera raised against the COOH-terminal peptide of GBF1. All sera showed similar staining of tubular elements adjacent to Golgi stacks (Fig. 9, A and B, brackets and arrows). These elements correspond to the regions of greatest antigenicity of COPI in rat liver sections (Fig. 9, C and

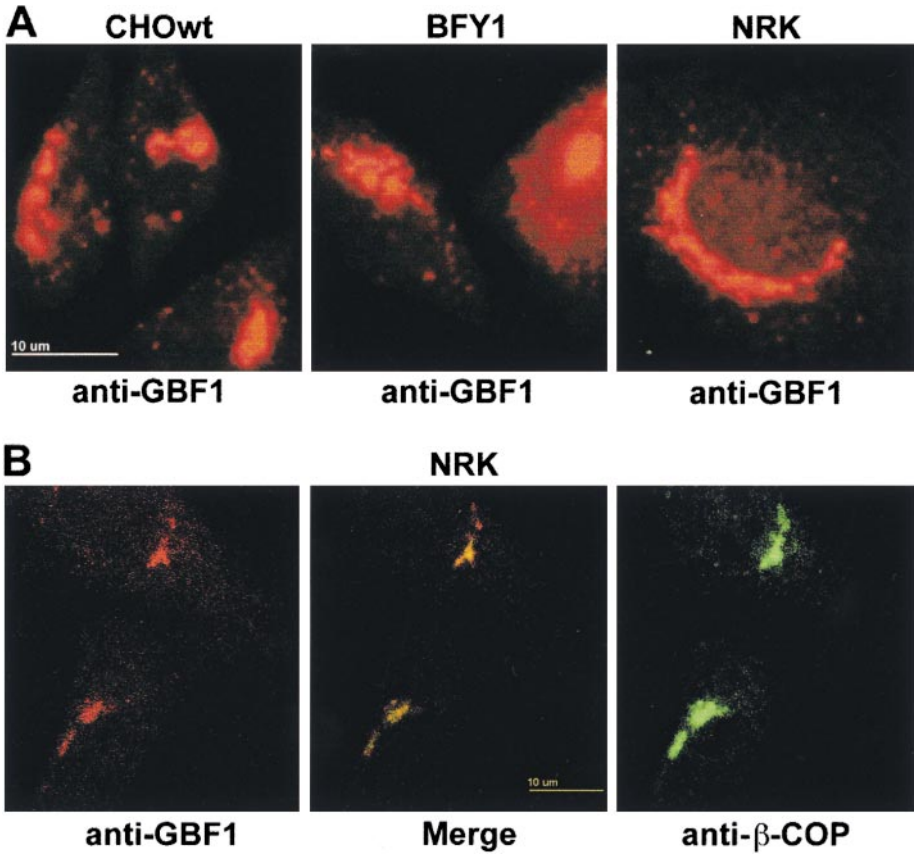

Figure 8. GBF1 colocalizes with β-COP to the Golgi complex. (A) Wild-type CHO (left), mutant BFY-1 (center), and NRK (right) cells were permeabilized and incubated with rabbit serum H154 (anti-GBF1) and stained with a Texas red–conjugated secondary antibody. Images obtained by standard epifluorescence microscopy are presented. (B) NRK cells were permeabilized and incubated with rabbit serum H154 (anti-GBF1) and mAb m3A5 (anti–β-COP) and stained with FITC- (β-COP) and Texas red– (GBF1) conjugated secondary antibodies. The images presented were obtained by confocal microscopy. (Left) GBF1 signal; (right) β-COP signal; and (center) merge of both images. Bars, 10 μm.

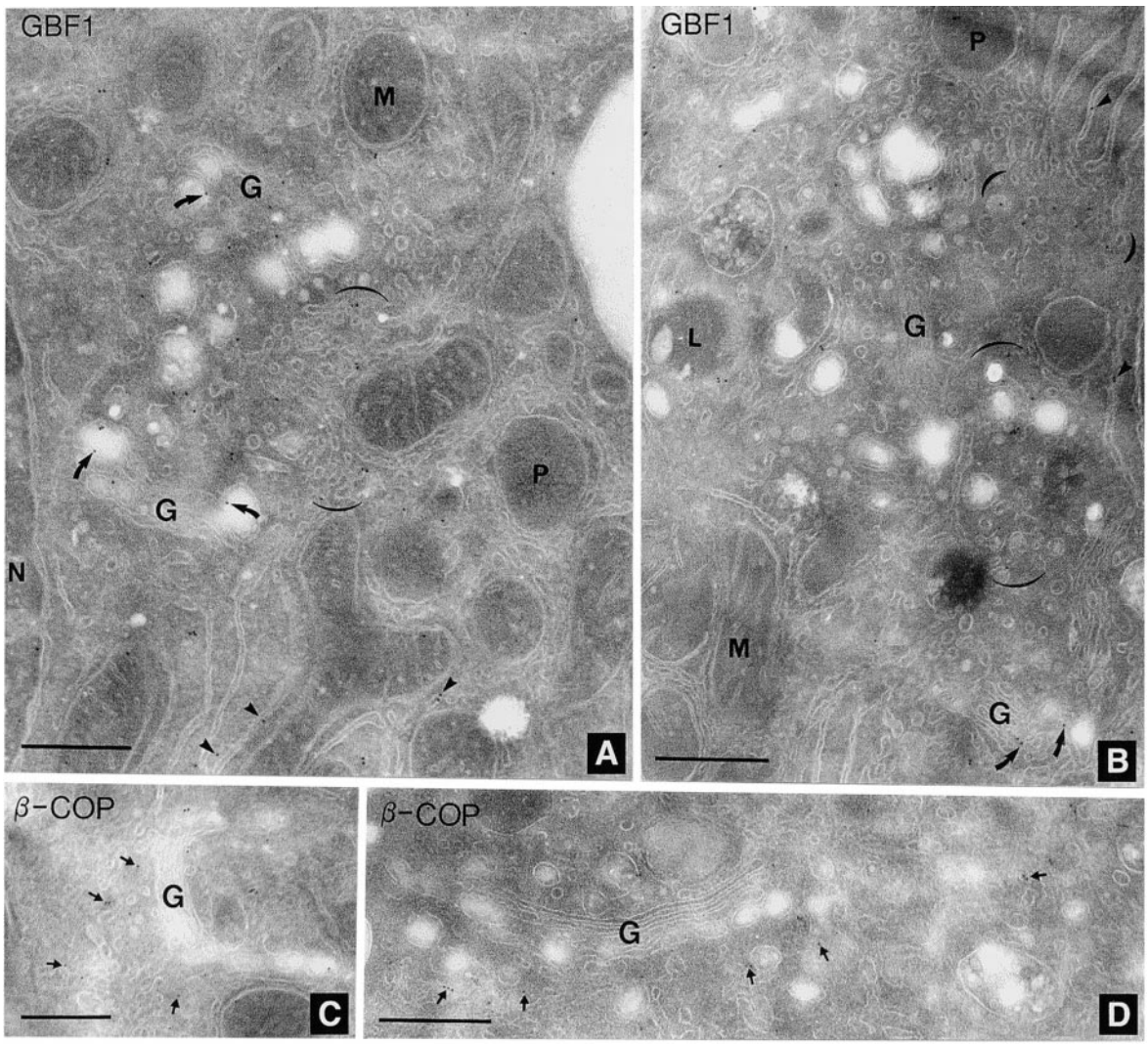

Figure 9. GBF1 localizes primarily to a tubular network proximal to Golgi stacks. Rat liver frozen cryosections were processed, labeled, and photographed as described in Materials and Methods. (A) anti-GBF1 serum H134 (diluted 1:2). (B) anti-GBF1 serum H133 (diluted 1:5). (C and D) mouse anti–β-COP (diluted 1:20). (Arrowheads) Gold particles labeling ER cisternae. (Arrows) Gold particles labeling tubular networks proximal to the Golgi complex. (Curved arrows) Gold particles labeling Golgi stacks. (Brackets) High concentration of gold particles labeling tubular networks proximal to the Golgi complex. M, mitochondria; P, peroxisome; and G, Golgi stack. Bars, 400 nm.

D; Dahan, S., and J.J.M. Bergeron, unpublished observations). Significant labeling was also observed over Golgi stacks, particularly at the electron lucent distensions (curved arrows), and on ER cisternae (arrowheads). Little staining of mitochondria and peroxisomes was observed under these conditions. Quantitative analysis of the GBF1 labeling experiments confirmed that even though a significant amount of GBF1 staining localized to peripheral tubules, the greatest concentration occurred in the Golgi region (Table II).

Discussion

An expression cloning strategy designed to identify proteins that promote Golgi-specific resistance to BFA, yielded a single cDNA from a library prepared from a highly BFA-resistant CHO mutant line. This cDNA encodes a novel 206-kD Sec7 domain protein, termed GBF1, that is primarily cytosolic and displays ARF-specific and BFA-resistant GEF activity. This protein localizes to the Golgi complex, displays specificity towards ARF5, and is a strong candidate for a GEF involved in regulating ARF activation for transport within the early secretory pathway.

Identification of a Novel BFA-resistant ARF-GEF

GBF1 was identified as a resistance factor that allowed growth in the presence of BFA. This activity appears dominant as cells transfected with the cDNA grew in the presence of BFA, and Golgi membranes recovered from these cells activated ARFs and recruited COPI in a BFA-resistant manner. As expected from the presence of a Sec7 domain, GBF1 displayed guanine nucleotide exchange activity that was clearly BFA resistant. Under physiological conditions, GBF1 appeared specific towards Group II ARFs since it exhibited clear GEF activity on ARF5 and

Table II. Labeling Density Distribution of GBF1

Compartment examined	Percent gold labeling	Labeling density
		gold/μm²
Golgi-stacked saccules	27.7	6.7
Golgi-associated tubules*	17.7	3.1
Distal tubules‡	38.3	3.2
Rough ER cisternae	16.2	2.5

35 micrographs of Golgi regions from ultrathin cryosections of rat liver immunolabeled with anti-GBF1 antibody (H133 diluted 1:5) followed by goat anti–rabbit IgG-10 nm gold were analyzed for the gold particle density in the indicated compartments. Only gold particles within 20 nm of a membrane profile were scored. Percentage gold particle labeling values represent the number of gold particles over respective compartments expressed as a fraction of the total number of gold particles (912) scored in the four secretory apparatus compartments examined. Labeling density values were obtained by scoring the total number of gold particles in a given compartment and dividing by the total profile area of that compartment. Mitochondrial and peroxisomal labeling density (2.0 gold particles/μm^2) in the same micrographs were used as an indicator of background labeling and were subtracted to yield the values presented.
*Includes tubular and vesicular profiles within 750 nm of a Golgi stack.
‡Includes tubule profiles at least 750 nm away from Golgi stacks.

much less towards ARF1/3. Although the role of ARF5 in protein transport and secretion remains unknown, it clearly associates with Golgi structures (Tsai et al., 1992) and this interaction is largely BFA resistant (Tsai et al., 1993). This BFA-resistant binding of ARF5 to Golgi membranes is consistent with the properties of GBF1 and further experiments will clarify the relationship between ARF5, GBF1, and the secretory pathway.

The sequence of the GBF1 Sec7d is not inconsistent with the observed BFA resistance of the GEF activity. Several recent reports demonstrate that Sec7d is a direct target of BFA since purified Sec7d from multiple proteins display an ARF-GEF activity that is BFA sensitive (Sata et al., 1998; Mansour et al., 1999; Peyroche et al., 1999). Analysis of the Sec7d sequence of a variety of proteins has recently established that variation on two key amino acids within motif 2 of this domain correlates with the BFA sensitivity of each family member (Peyroche et al., 1999). The consensus motif for BFA-sensitive proteins is LS**YS**IIMLNTDL and that for BFA-resistant proteins is LS**FA**IIMLNTSL. Mutation of FA to YS is sufficient to convert the ARF-GEF activity of the Sec7 domain of ARNO from BFA resistant to BFA sensitive. The fact that motif 2 of GBF1 displays a hybrid sequence (LA**YA**VIMLNTDQ; Fig. 3 A) and contains three additional amino acids (NVP), eight residues downstream of motif 2 relative to the consensus sequence, may account for the BFA resistance and ARF specificity of this protein.

Since all previously characterized large Sec7d proteins display BFA-sensitive GEF activity (Peyroche et al., 1996; Morinaga et al., 1997), we expected the BFA resistance of GBF1 to result from mutation of a wild-type BFA-sensitive GEF. However, cloning of GBF1 cDNAs from wild-type CHO cells and further characterization of BFY-1 transcripts established that processing variants in those two lines have identical sequences. Furthermore, transfection of GBF1 cDNAs recovered from the wild-type library promoted growth in the presence of BFA. The BFA-resistant phenotype caused by GBF1 in transformed 293 cells must, therefore, result from overexpression of a naturally BFA-resistant protein. The observation that GBF1 levels were identical in the parental CHO and mutant BFY-1 lines, indicated that resistance in the mutant line arose from changes in a different gene product such as BFA-sensitive GBF or p200 isoforms. Our inability to identify the BFY-1 resistance factor could have resulted from the fact that this protein is part of a complex or that its cDNA is present at very low levels in the library. Alternatively, resistance in BFY cells could have arisen from mutations in more than one gene. Ongoing analysis of our BFA-resistant lines should elucidate the resistance mechanism.

GBF1 Is a Strong Candidate for a Regulator of ARF Activation during Traffic through the Early Exocytic Pathway

GBF1 manifests many of the properties of an ARF-GEF implicated in recruitment of COPI for traffic within the early exocytic pathway. First, transfection with its cDNA yielded Golgi membranes displaying normal levels of GBF1 and ARF-GEF activity that recruited COPI in a BFA-resistant manner. In addition, as expected of such an ARF-GEF, it colocalized with COPI to the Golgi complex and smooth tubules that may correspond to traffic intermediates between the ER and Golgi complex. Finally, the fact that its sequence is most similar to that of Gea1p/Gea2p further supports this model. Overexpression of either of these proteins, but not that of Sec7p, was found to suppress the dominant-negative effects of an ARF1 mutant that has reduced nucleotide binding capacity and is thought to block growth by sequestering its GEF (Peyroche, 1996).

The present experimental evidence is insufficient to define the exact role of GBF1 since it does not reveal in which direction of transport this GEF functions. The fact that BFA blocks anterograde traffic suggests that GBF1 does not normally function as the primary GEF responsible for coat recruitment for anterograde traffic. However, since retrograde traffic from the Golgi is not blocked by BFA, the naturally BFA-resistant GBF1 may well function primarily in that direction. Our results indicate that the overlap in function between GBF1 and the BFA-sensitive GEF involved in anterograde traffic is, nevertheless, sufficient to allow BFA-resistant forward traffic when GBF1 is overexpressed. This could occur by partially displacing the BFA-sensitive GEF, possibly p200 (Mansour et al., 1999), which normally regulates this process. This displacement of the normal GEF by GBF1 would explain the cytotoxicity resulting from gross overexpression after transfection of undiluted pCEP4-GBF1 (see Materials and Methods).

In contrast to Sec12p, the membrane-associated GEF for Sar1p responsible for COPII recruitment (Barlowe et al., 1994), GBF1 is primarily soluble. This suggests that this GEF is recruited only when and where needed to initiate budding. Such a regulated recruitment mechanism for a retrograde-specific GEF may be necessary if Golgi cisternae were not stable structures, but rather were constantly remodeled during the cisternal progression for which experimental evidence has been accumulating (Bonfanti et al., 1998; Glick and Malhotra, 1998). The observation that normal levels of GBF1 and ARF-GEF activity were associated with Golgi membranes despite a five- to eightfold overexpression indicates that the number of recruitment

sites may be limited. In such a model of GBF1 action membrane recruitment would most likely be accompanied by GEF activation.

Sec7d Proteins: A Family of ARF-GEFs?

The Sec7d family can be broadly separated in three classes that may have distinct but related functions. The highly similar small members (~50 kD) were represented in Fig. 2 B by the better characterized ARNO protein. The larger and more heterogeneous members (160–210 kD), all listed in that figure, can be divided into two classes. Sequence comparison identified up to 14 regions of highly conserved sequence outside the defining Sec7 domain. The small ARNO-like proteins contain only the Sec7d and constitute a class of their own. As shown in Fig. 2 B, GBF1, Gea1/2, and to a lesser extent EMB30 can be grouped in a class based on the number of shared domains. Sec7 and p200 form an apparently distinct group by default based on their low number of shared domains. At present it is not clear whether these two proteins will give rise to separate subclasses, but it is likely that this preliminary classification will be refined as more members and/or isoforms of the Sec7d family are discovered in higher eukaryotes.

The specific functions of various members of this family in protein traffic are likely determined by their specificity towards various ARFs and the intracellular membranes to which they are recruited. For example, small members of this family such as ARNO localize to the endosomal compartment and appear specific for ARF6 (Frank et al., 1998). It is interesting to note that these small Sec7d proteins all contain a pleckstrin homology domain. Such domains bind phosphoinositides (Harlan et al., 1995; Rebecchi and Scarlata, 1998) and facilitate recruitment of the GEF to the membrane where it can act more effectively on its membrane-associated substrate (Chardin, 1996). The large members of the family lack this domain and may utilize a more complex mechanism to regulate membrane association through their additional domains. Interestingly, the various Sec7d proteins, being potential targets for BFA, could constitute the multiple organelle specific targets whose existence was suggested by earlier studies.

GBF1 and p200 both localize to the Golgi complex (this work and Mansour et al., 1999) and mutations in their orthologues Gea1p/Gea2p and Sec7p were found to interfere with protein secretion (Achstetter et al., 1988; Peyroche et al., 1999). Both classes of large Sec7d proteins are, therefore, implicated in the exocytic pathway. Our demonstration that these two classes of ARF-GEF may act on groups I and II ARFs selectively and, thus, have distinct specialized functions may provide a mechanism to explain how COPI could be involved in both anterograde and retrograde traffic. Further studies on GBF1 and p200 and the proteins with which they associate are certain to shed more light on these somewhat contradictory roles of ARF and COPI in movement of proteins to and from the Golgi complex.

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