Isolation and Characterization of Mutant CHO Cell Lines with Compartment-specific Resistance to Brefeldin A

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Abstract. 22 CHO[BFY] (BFY) cell lines were isolated at a frequency $2-30 \times 10^{-7}$ from mutagenized populations on the basis of their ability to grow in the presence of 1 μg/ml brefeldin A (BFA). Four of the five mutant lines tested are genetically stable and none of the mutant lines characterized degrade this drug. Immunofluorescence studies reveal that whereas early endosomes and the Golgi complex have nearly identical BFA sensitivities in the parent CHO line, the relative sensitivities of these two organelles were dramatically altered in all six mutant lines tested. Four cell lines maintain normal Golgi appearance at a BFA concentration as high as 10 μg/ml. Mutant lines show wide variation in the level of resistance to growth inhibition by BFA, but none of the mutant lines characterized grow above 2 μg/ml BFA. This specific growth inhibi-

tion is observed under conditions where Golgi morphology and function remain unaffected, suggesting that some factor(s) unrelated to Golgi function remains sensitive to BFA in BFY mutant lines. These observations provide strong evidence for the presence of multiple, organelle-specific targets for BFA. Cell-free measurements with membrane extracts establish that resistance to BFA in BFY-1 cells involves a membrane-associated factor distinct from ARFs and coatomers. This collection of mutant lines may prove valuable for the identification of intracellular target(s) for BFA and/or of effectors that interact upstream or downstream with these targets, thereby uncovering the cascade which regulates assembly of organelle-specific coats.

BREFELDIN A (BFA)[1] is a lipophilic fungal metabolite that blocks protein secretion (Misumi et al., 1986) and induces rapid and profound morphological changes in several organelles of the secretory pathway (reviewed in Pelham, 1991; Klausner et al., 1992). Remarkably, these effects are completely reversible (Donaldson et al., 1990). These properties make BFA a unique and powerful tool to unravel the mechanisms that underly organelle structure and identity.

In many different cell types, BFA causes disassembly of the Golgi complex and redistribution of Golgi resident enzymes into the ER (reviewed in Klausner et al., 1992). This transformation prevents normal transport of proteins from the ER and results in a block of protein secretion. The resorption of Golgi cisternae into the endoplasmic reticulum occurs by extension of tubules which subsequently fuse with the ER, a process greatly enhanced by microtubules (Lippincott et al., 1990). Although it is often assumed that BFA

causes the complete disappearance of the Golgi complex, the presence of vesicle clusters and tubules containing Golgi markers, or Golgi "remnants," has been documented in several cell lines (Ulmer and Palade, 1991; Hendricks et al., 1992; Hidalgo et al., 1992). The reorganization of the Golgi complex caused by BFA can be detected as a dramatic decrease in the rate of processing of carbohydrate side chains which normally occurs as glycoproteins transit through the individual cisternae of the Golgi (Klausner et al., 1992 and references therein).

The effects of BFA are not limited to the Golgi complex. Treatment with BFA causes extensive tubulation and fusion of the early endosome (EE) and the TGN to form a hybrid organelle (TGN/EE), clustering in the form of tubulovesicular elements near the microtubule-organizing center (Lippincott et al., 1991; Tooze and Hollinshead, 1992; Wood and Brown, 1992). Limited tubulation of lysosomes also results from BFA treatment in some cell types (Lippincott et al., 1991; Wood et al., 1991), but these tubular lysosomes remain separate from the TGN/EE hybrid. These morphological transformations appear to have no significant effect on the ability of cells to recycle transferrin and extract iron (Lippincott et al., 1991). Transfer of material from EE to lysosomes also continues in most cell types in spite of these morphological changes (Wood et al., 1991).

Several lines of evidence suggest that the effects of BFA on

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1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; BFA, brefeldin A; EE, early endosome; endo-H, endoglycosidase H; hTf, human transferrin; Man II, mannosidase II; VSV, vesicular stomatitis virus; VSV-G, envelope glycoprotein of VSV.

organelles most likely involves an interaction with a specific target protein. First, closely related structural analogues of BFA are completely inactive, including 7-epi- and 4,7-diacetyl-10-epoxy-derivatives of BFA (Orci et al., 1991; Donaldson et al., 1992; Klausner et al., 1992). Second, BFA treatment of a heterokaryon resulting from the fusion of a BFA-sensitive cell (CV-1) with a naturally occurring BFA-resistant cell (PtK1) does not affect the Golgi originating from the resistant variant while completely disassembling the Golgi from the sensitive cell (Ktistakis et al., 1991). These observations strongly suggest that the target for BFA is a protein, and that it is most likely membrane associated.

A significant step towards understanding the mechanism of action of BFA was made when it was established that BFA interferes with assembly of cytoplasmic coats on Golgi membranes. Two main coat protein complexes have been characterized in detail, the classic clathrin coat found on coated endocytic pits on the cytoplasmic face of the TGN, and a distinct nonclathrin coat observed on vesicles associated with the Golgi complex (Melançon et al., 1991; Klausner et al., 1992; Rothman and Orci, 1992). BFA has been shown to prevent association of β-COP-containing coatomer with Golgi membranes both in vivo and in vitro (Donaldson et al., 1991a, b). Interference with coat assembly may be a general mechanism to explain the effects of BFA on all membrane systems. Indeed, BFA has also been shown to prevent assembly of clathrin-coated vesicles driven by Golgi-localized adaptor complexes (Robinson and Kreis, 1992).

Recent evidence suggests that the effect of BFA on coat assembly may be, in part, a consequence of interfering with the association of ADP-ribosylation factor (ARF) with the Golgi membranes. ARFs are small GTPases required for both the assembly of coat proteins on Golgi membranes (Donaldson et al., 1991a, 1992; Palmer et al., 1993) and the formation of transport vesicles on Golgi cisternae (Taylor et al., 1994). The exchange of GTP for GDP on ARFs promotes its membrane association (Kahn, 1991) and BFA was found to interfere with this nucleotide exchange activity in vitro on recombinant ARF (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993). However, this effect may be indirect. Members of the heterotrimeric G-protein family appear to be also involved in coat assembly, possibly by regulating guanine nucleotide exchange on ARFs (Bomsel and Mosto, 1992; Melançon, 1993). It has been shown that pretreatment with agents that activate G-proteins specifically (i.e., without effects on ARFs or other members of the ras superfamily), such as aluminum fluoride and bacterial toxins, can prevent the effect of BFA on the association of β-COP with Golgi membranes (Donaldson et al., 1992; Ktistakis et al., 1992). These observations may suggest that G-proteins or their activators are the true cellular target(s) of BFA.

Comparison with various cell lines derived from a variety of tissues and species (Pelham, 1991) showed that BFA affects many cell lines in an organelle-specific manner. For example, whereas BFA blocks protein exit from ER-Golgi systems in most cell lines, it has no such effect in MDCK (Hunziker et al., 1991) or PtK1 cells (Ktistakis et al., 1991). Also, an apparent block in transport to lysosomes reported in chick embryo fibroblasts (Lippincott et al., 1991) is not observed in either NRK cells, bovine testicular cells (Wood and Brown, 1992), or rat hepatocytes (Misumi et al., 1986).

These observations, although suggestive, do not establish that the organelle-specific effects of BFA result from interaction of BFA with distinct, organelle-specific, targets. For example, different organelle-specific effects between various cell lines could be obtained if the activity of a unique BFA target were required to different extents by the various endomembrane systems in a species- or tissue-dependent manner.

In this study, 22 CHO[BFY] mutant lines were isolated on the basis of their ability to grow in the presence of BFA. We find that whereas early endosomes and the Golgi complex have nearly identical BFA sensitivities in the parent CHO line, the relative sensitivities of these two organelles were dramatically altered in all mutant lines tested. This observation suggests that the organelle-specific effects by BFA in our mutant lines are unlikely to arise from the differential requirement of the activity of a unique BFA target in different organelles and thus provides strong evidence for the presence of multiple cellular targets for BFA. Cell-free measurements with membrane extracts establish that resistance to BFA in BFY-1 cells involves a membrane-associated factor distinct from ARFs and coatomers and therefore suggest that resistance in these cells could have occurred by mutation of the BFA target itself. The availability of these CHO[BFY] mutant lines derived from a single parent line and demonstrating organelle-specific BFA resistance will provide important tools to identify and characterize the multiple molecular interactions leading to assembly of organelle-specific protein coats.

Materials and Methods

Reagents

BFA was purchased from either Epicentre Technologies (Madison, WI) or Sigma Chemical Co. (St. Louis, MO), and was stored at $-20°C$ as a stock solution of 5 mg/ml in either 100% ethanol or DMSO. BFA analogues, B27 and B36, were generous gifts from Dr. J. Donaldson (NIH, Bethesda, MD) and were stored at $-20°C$ in DMSO. Endoglycosidase H (endo-H), ATP, UTP, creatine phosphate, rabbit creatine phosphokinase, and iron-saturated human transferrin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Unless otherwise indicated, all other biochemicals were obtained from Sigma Chemical Co.

Antibodies

The m3A5 mouse monoclonal antibody which recognizes the 110-kD β-COP protein was generously supplied by Dr. Thomas Kreis (University of Geneva, Switzerland). A rabbit antiserum raised against Golgi mannosidase II (Man II) was kindly provided by Drs. K. Moremen (University of Georgia, Athens, GA) and M. Farquhar (University of California-San Diego, LaJolla, CA). Rabbit anti–human transferrin antibody, rhodamine-and FITC-labeled goat anti–mouse, and goat anti–rabbit IgG were obtained from Boehringer Mannheim Biochemicals and used at 1:400 for antibody to transferrin and 1:200 dilution for fluorophore-labeled antibodies. A mouse monoclonal antibody against the envelope glycoprotein of vesicular stomatitis virus (VSV-G) was prepared by chromatography over protein-A Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) of supernatant of cultures of hybridoma clone 8G5 (LeFrancois et al., 1982), a generous gift of Dr. LeFrancois (Upjohn Company, Kalamazoo, MI).

Cell Culture, Cytosol, and Golgi-enriched Membrane Preparation

The parent line for our studies, CHO[pro−5] (American Type Culture Collection, Rockville, MD), is a derivative of line CHO-S, a cell line adapted for growth in Suspension culture. The terms CHO[pro−5] and wild-type CHO will be used interchangeably in this paper. CHO[pro−5] and CHO[BFY] mutant lines were maintained in α-MEM supplemented with 7.5% fetal calf serum,

100 μg/ml penicillin G, and 100 μg/ml streptomycin. Vesicular stomatitis virus (VSV), Indiana strain, was propagated in BHK cells and stored as culture supernatant in small aliquots at −70°C. Golgi-enriched membranes and cytosol were prepared from crude homogenates of CHO cells as described (Balch et al., 1984).

EMS Mutagenesis of CHO^{pro-5} (Wild Type) Cell Line

CHO^{pro-5} cells were plated in α-MEM medium supplemented with 7.5% fetal bovine serum at a concentration of 2×10^6 cells/150-mm petri dish. After 6 h, cells were treated with 300 μg/ml of ethyl methanesulfonate (Aldrich Chemical Co., Milwaukee, WI) for 16 h to a survival level of 75%. Thymidine at a concentration of 2 μg/ml was included to increase mutagenesis efficiency (Peterson et al., 1978). Cells were then cultured for a total of 3 d before challenge with 1 μg/ml of BFA. Colonies which appeared 1–2 wk after BFA treatment were cloned and cultured in the presence of 0.5 μg/ml BFA for several days before freezing and storage in liquid N_2.

Cell Growth Assay

The cell growth assay was modified from the method of Bodary (Bodary et al., 1989). Cells from either wild-type or mutant lines were plated in 96-well tissue culture plates (6–8 × 10^3 cells per well) and treated with concentrations of BFA from 0 to 2.0 μg/ml. After 48 h of culture (enough time for control wells to reach near confluency), the plates were washed with PBS and fixed in 10% formaldehyde for 30 min. Each well was then treated with 0.5% crystal violet in 20% methanol for 10 min (Bodary et al., 1989) and washed three times with ddH$_2$O to remove free dye. Bound dye was released from the cells with 50 μl 1% SDS in distilled water. The absorbance at 595 nm was determined using an ELISA plate reader (Vmax; Molecular Devices, Menlo Park, CA). Control experiments established that the absorbance at 595 nm is proportional to the number of cells present in each well. Each assay was done in triplicate, and repeated four or more times.

Labeling of Early Endosomes and Immunofluorescence Microscopy

CHO cells were grown on coverslips (No.1). Early endosomes were labeled by incubating cells first with BSA (5 mg/ml) in α-MEM for 30 min, and then with 10 μg/ml of iron-saturated human transferrin (hTf) for 1 h at 37°C as described (Lippincott-Schwarz et al., 1991). After the various treatments indicated in the text, cells grown on coverslips were washed briefly with PBS, fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and then permeabilized with 0.1% TX-100 and 0.05% SDS in PBS for less than 4 min. After several washes with PBS, cells were incubated with primary antibody in PBS for 45 min to 1 h, washed, and incubated for 45 min to 1 h with rhodamine- or FITC-conjugated secondary antibodies. After a brief rinse with water, the cells were mounted and viewed on a microscope equipped for epifluorescence (M35; Karl Zeiss, Oberkochen, Germany) (Fig. 6) or for confocal microscopy (MultiProbe 2001; Molecular Dynamic Corp, Sunnyvale, CA) (see Fig. 5).

Thin Layer Chromatography of [^{3}H]BFA

7-[^{3}H]BFA (3.2 Ci/mmol; a generous gift of Dr. F. Wieland, University of Heidelberg, Germany) was added to 100 μl suspensions (final BFA concentration ∼0.3 μg/ml) containing 1×10^6 cells of either the wild type or various mutant lines in α-MEM supplemented with 10 mM Hepes (pH 7.4). Following a 5-h incubation at 37°C, the cells were then separated from incubation medium by centrifugation and the cell pellets were extracted with 20 μl methanol/water (1:1). 5-μl aliquots of the incubation medium and cell extract were subjected to thin layer chromatography on silica gel 60 plates (E. Merck, Darmstadt, Germany). The TLCs were developed using one of two different mobile phases: chloroform/methanol (80:20), or chloroform/methanol/water/formic acid (10:10:3:0.6) which yield R_f values for BFA of 0.5 and 0.9, respectively. TLC plates were treated with Amplify (Amersham Corp., Arlington Heights, IL) as per manufacturer's instructions, dried, and exposed to x-ray autoradiographic film for fluorography. The fluorograms were quantitated by densitometry (ULTRO-Scan laser densitometer; LKB, Uppsala, Sweden). Quantitation demonstrated that the fraction of BFA associated with the cell pellet was consistantly small (∼10%) in wild type and in all BFY lines tested, whether measured after a short (1 h) or long (5 h) incubation.

Analysis of VSV-G Glycoprotein Processing

Subconfluent monolayers of mutant BFY-1 or wild-type CHO^{pro-5} cells growing in six-well culture plates were infected with VSV for 2 h 45 min, washed free of unbound virus, and then incubated for 30 min in the presence or absence of 2 μg/ml BFA in DME medium free of methionine. Cells were then pulse labeled for 15 min with 20 μCi/well of [^{35}S]methionine (Trans-^{35}S-label; ICN Biochemicals, Inc., Irvine, CA) at 37°C. At the end of the pulse, the radioactive medium was removed and the cells were chased in complete medium containing a 10^4 molar excess of cold methionine for 0 or 60 min, in the absence or presence of 2 μg/ml BFA. The chase was terminated by placing the cells on ice. Cells were then lysed with 0.25 ml lysis buffer (50 mM Tris [pH 7.5], 1% Triton X-100, 1% DOC, and 1 mM EDTA). Nuclei were removed by could centrifugation for 10 min. VSV-G antibody was then incubated with 100-μl samples for 1 h on ice followed by incubation with protein-A Sepharose 4B fast flow (Sigma Chemical Co.) (1:1) in lysis buffer. The precipitates were recovered by centrifugation, washed three times with cold lysis buffer, resuspended in 20 μl of 0.8% β-mercaptoethanol and 0.1% SDS in 0.1 M sodium acetate (pH 5.5), and heated for 5 min at 95°C. Samples were then divided into two equal aliquots. For endo-H digestion, one aliquot was incubated with one milliunit of endo-H per 30-μl reaction volume for 16 h at 37°C. The digestion was terminated with addition of Laemmli sample buffer. Samples were heated at 90°C for 5 min before loading onto 10% SDS-gel.

Quantitation of ARFs, β-COP, and Binding of Coatomers to Golgi Membranes

The relative amounts of ARFs and β-COP in cellular extracts were determined using GTP-ligand blots and immunoblots, respectively. For ARF detection, GTP-ligand blots were used since they are significantly more sensitive than immunoblots. GTP-ligand blots of total cellular extracts, cytosol, and Golgi-enriched membrane fractions were carried out essentially as described (Taylor et al., 1994) and quantitated using a PhosphorImager (Molecular Dynamics Corp.). For determination of β-COP levels, proteins transferred to nitrocellulose were probed with monoclonal antibody m3A5 and detected by enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL). The results were quantitated by densitometry (ULTRO-Scan laser densitometer; LKB). Membrane association of coatomers was measured using a β-COP binding assay which contained 90 μg of cytosol prepared either from BFA-sensitive CHO cells and 10 μg of Golgi-enriched membranes prepared either from BFA-sensitive CHO^{pro-5} cells or from mutant BFY-1 cells in a final volume of 200 μl. The assay was carried out in a buffer containing 25 mM Hepes (7.4), 50 mM KCl, 2.5 mM MgCl$_2$ and an ATP-regenerating system (8 U/ml creatine phosphokinase, 5 mM creatine phosphate, 100 μM ATP, and 100 μM GTP). Following a 20-min incubation in the presence or absence of BFA at 30°C, membranes were collected by centrifugation and resuspended in SDS sample buffer. All of each sample was loaded on 10% SDS gel and the relative amounts of membrane-associated β-COP quantitated by Western blot as described above.

Results

Selection of BFA-resistant CHO Cell Lines

Preliminary studies with the parent cell line CHO^{pro-5} (hereafter referred to as wild type) showed that BFA at concentrations as low as 0.2 μg/ml inhibited growth of wild-type CHO cells. Addition of the drug to cells grown in monolayers caused the cells to round up within 12 h and to completely detach from the substratum after 48 h of continuous exposure. This demonstrated the feasibility of selecting resistant cell lines by addition of drug to the growth medium.

BFA-resistant colonies were selected by challenging ethyl methanesulfonate (EMS)-mutagenized CHO monolayers with 1 μg/ml BFA. Resistant colonies appeared after 1–2 wk of continuous growth. Three such selections yielded 22 individual cell lines at frequencies varying from 2–30 colonies per 10^7 mutagenized cells (Table I). These cell lines were labeled BFY-1 to BFY-22. The frequency at which these mu-

Table I. Results of Isolation of CHO Mutant Lines Resistant to Brefeldin A

Mutant cell lines	Time for colony appearance	Frequency mutants/10^7 cells
BFY-1 to BFY-3	1 wk	2–4
BFY-4 to BFY-6	2 wk	≈30
BFY-7 to BFY-22	2 wk	15

CHO^{pro-5} cells were treated with 300 μg/ml EMS to a survival level of 75%. Cells were then cultured in the absence of EMS for 3 d before replating in medium containing 1 μg/ml of BFA, as described in Materials and Methods. Three separate selections were carried out. The time and frequency at which colonies appeared in each case are listed.

tant lines were obtained suggests that although there might be multiple targets for BFA, mutation in a single gene is sufficient to allow growth under our selection conditions. Recent reports on the isolation of a mutant Vero and two mutant human carcinoma lines which can grow in the presence of BFA support this interpretation (Chen et al., 1992; Seguchi et al., 1992).

Cell Lines Vary Greatly in the Level of Resistance to BFA

The mutant cell lines were first characterized with respect to their resistance to growth inhibition in the presence of BFA. A growth assay was performed as described in Materials and Methods. Using this assay, the growth properties of wild type and 10 randomly chosen mutant lines were
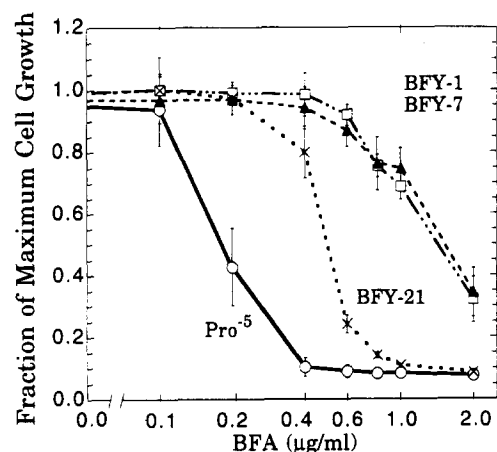

Figure 1. The growth of mutant CHO^{BFY} cell lines display varied and distinct extents of resistance to BFA. Equivalent amounts of cells from each cell line to be tested (6–8 × 10^3 cells/well) were delivered to each well in a 96-well tissue culture plate. BFA was administered at increasing concentrations from 0 to 2.0 μg/ml and added right after plating. As controls, one well was left untreated while another received an appropriate amount of DMSO, the solvent used to dissolve BFA. The number of cells remaining attached after 48 h was evaluated using the cell growth assay described in Materials and Methods. BFA-sensitive wild-type CHO^{pro-5} cells were used as a control in each experiment. Each line was tested in triplicates and the results are expressed as fraction of maximum cell growth relative to the OD_{595} measured for cells grown in the absence of BFA. Four to five such titrations were carried out for each cell line and the average values with error (standard deviation) were plotted in a semi-log scale.

Table II. Results of Growth Assays on Subset of the BFY Mutant Lines

Mutant line	LD_{50}* μg/ml
Pro-5	0.2
BFY-11	0.4
BFY-21	0.5
BFY-15	0.8
BFY-16	0.8
BFY-9	1.3
BFY-1	1.6
BFY-2	1.6
BFY-3	1.7
BFY-7	1.7
BFY-12	>1.0

* Lethal dose 50%. Growth assays at several BFA concentrations were carried out for each cell line as described in legend to Fig. 1. The concentration of BFA which causes a 50% reduction in cell density after 48-h incubation, or LD_{50}, is listed for each cell line tested.

compared. The doubling times for growth of all mutant lines in the absence of BFA are very similar and nearly identical to that of the parent line. The effect of BFA on the growth of a subset of these lines is shown in Fig. 1. The concentrations of BFA required to cause a 50% decrease in cell density (LD_{50}) after two days of culture were determined for each cell line based on at least four separate titrations with BFA and are given in Table II. These results demonstrate that mutant cell lines are indeed resistant to BFA compared to the parent wild-type line. Furthermore, we find that BFY mutant lines vary greatly with respect to their tolerance for BFA in the growth assay. Differences in the mechanism of resistance among the mutant lines could account for this variation.
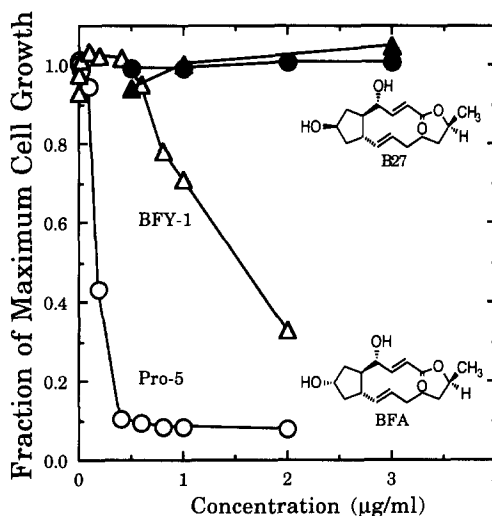

Figure 2. Growth inhibition by BFA observed in mutant lines is specific. Wild-type and the mutant BFY-1 cell lines were used for a cell growth assay as described for Fig. 1. Increasing amounts of either BFA or its analogue, 7-epi-BFA (*B27*) were added to successive wells. The extent of growth measured after 48 h is plotted as a function of drug concentration. Treatment with the analogue B27 (*solid lines*) has no growth inhibition on both wild-type and BFY-1 mutant lines. Similar results were obtained with another inactive analogue, 4,7-diacetyl-10-epoxy-BFA (*B36*) (data not shown).
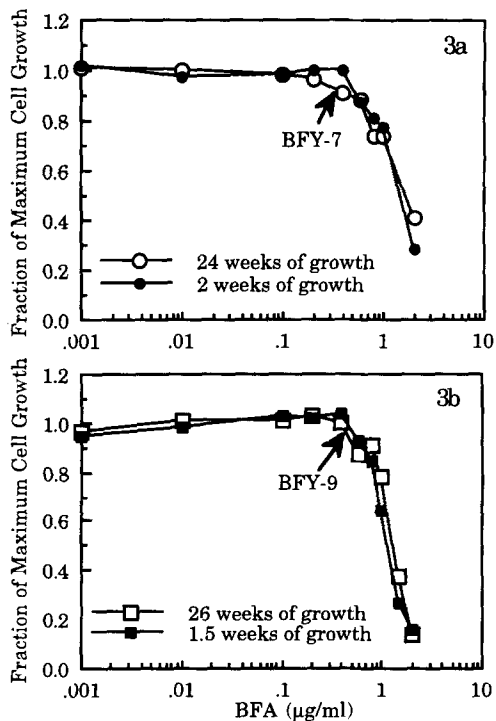
Figure 3. The BFA resistance phenotype is genetically stable. Mutant BFY-7 and BFY-9 grown for different lengths of time in absence of BFA were used for cell growth assays similar to those presented in Fig. 1. The duration of culture in absence of BFA is indicated.

Growth Inhibition by BFA Observed in Mutant Lines Is Specific

Despite the fact that mutant lines are clearly resistant to BFA relative to wild-type CHO cells, growth inhibition is ob-

served at BFA concentrations above 1 μg/ml in all mutant lines tested and none of the mutant lines grow at BFA concentrations above 2 μg/ml (Fig. 1). To determine whether this growth inhibition at high BFA concentration results from nonspecific effects, several BFA inactive analogues were tested in the cell growth assay. As shown in Fig. 2, a 7-epimer of BFA, also called B27, had no effect in this assay either on wild-type or on mutant BFY-1 cells at concentrations as high as 3.0 μg/ml. Similar results were obtained (data not shown) with another BFA analogue, 4,7-diacetyl-10-epoxy-BFA, or B36, which has also been shown to be inactive in displacing β-COP from Golgi membranes (J. Donaldson, personal communication).

The BFA-resistant Phenotype Is Genetically Stable in Most Lines Tested

The stability of the phenotype was tested by comparing the growth properties of several lines following prolonged culture in the absence of the drug. If resistance results from a stable genetic change (as opposed to transient gene amplification), we would expect the LD_{50} for a given line to remain unaffected by extensive culture in the absence of drug. The results obtained with two cell lines, BFY-7 and BFY-9, grown in the absence of BFA for less than two or greater than 24 wk are shown in Fig. 3. Clearly, culture for up to 26 wk in the absence of BFA caused no reduction in the level of resistance. Similar results were obtained for the BFY-1 and BFY-12 lines.

Resistance to BFA Does Not Result from Drug Degradation

Previous studies by F. Wieland and colleagues on the stability of BFA in CHO cells have established that BFA can be metabolically deactivated, primarily via conjugation with
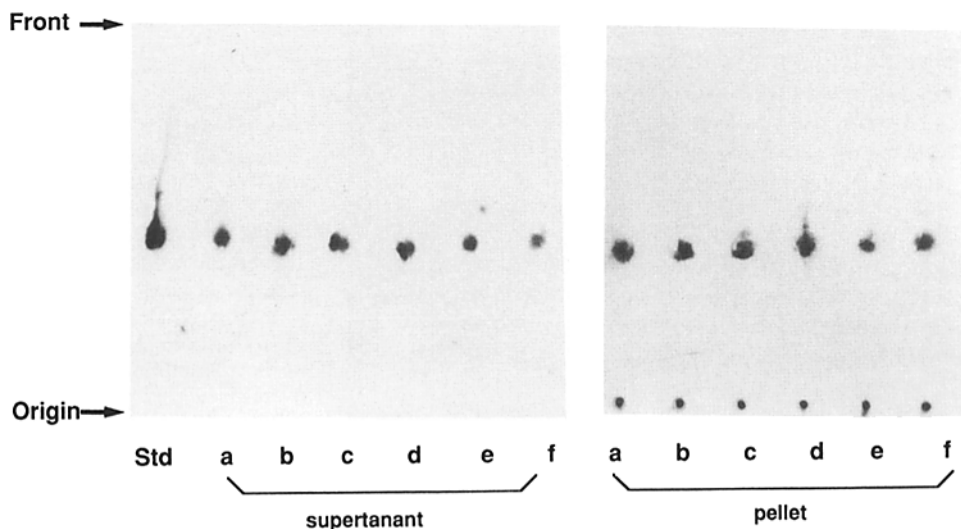

Figure 4. Resistance to BFA in mutant cell lines tested is not due to BFA degradation or modification of the drug by mutant cells. CHO[pro-5] cells and mutant cells were incubated with [³H]BFA in α-MEM containing 10 mM Hepes (pH 7.4) to give final concentration of 0.3 μg/ml [³H]BFA. Samples a–f correspond to wild type, and BFY-1, -7, -11, -12, -21, respectively. Following a 5-h incubation, the cells were separated from medium by centrifugation. Small aliquots of medium and cell pellet (dissolved in 50% methanol) were subjected to TLC as described in Materials and Methods.

The solvent used for this experiment was optimized to resolve BFA with an R_f value of 0.5. Fluorograms of the TLC plates are shown and arrows indicate the position of the origin and solvent front. A fivefold greater amount of cell pellet material (right) relative to medium (left) was loaded and the TLC for the pellet fractions was exposed to film three times longer. Fluorograms were quantitated by densitometry. A threefold increase in exposure did not lead to detection of any other spots. Quantitation demonstrated that the fraction of BFA associated with the cell pellet was consistantly small (~10%) in wild type and in all BFY lines tested, whether measured after a short (1 h) or long (5 h) incubation.

glutathione (Brüning et al., 1992). We therefore measured whether degradation is enhanced in BFY mutants, and established that no significant degradation of BFA is observed in all five of the mutant lines tested.

The metabolism of 7-[³H]BFA in either wild type or various mutant lines was investigated as described in Materials and Methods. The results of thin layer chromatography analysis of medium and cell extracts prepared after a 5-h incubation with 7-[³H]BFA are presented in Fig. 4. No BFA degradation products were observed in any of the cell lines tested, either in the cell pellet or in the medium. Similar results were obtained using another solvent system with a very different R_f value (data not shown). The lack of sig-

nificant degradation or modification of BFA observed here is inconsistent with the slow (36 ng h⁻¹/10⁶ cells) modification of BFA previously reported (Brüning et al., 1992). This difference could result from the use of different CHO isolates in the two studies. Resistance to BFA might also have arisen from lack of import or increased export of the drug. These possibilities are eliminated by experiments presented below in Fig. 6.

The Morphology of the Golgi Complex in Mutant Cells Is Stable to BFA

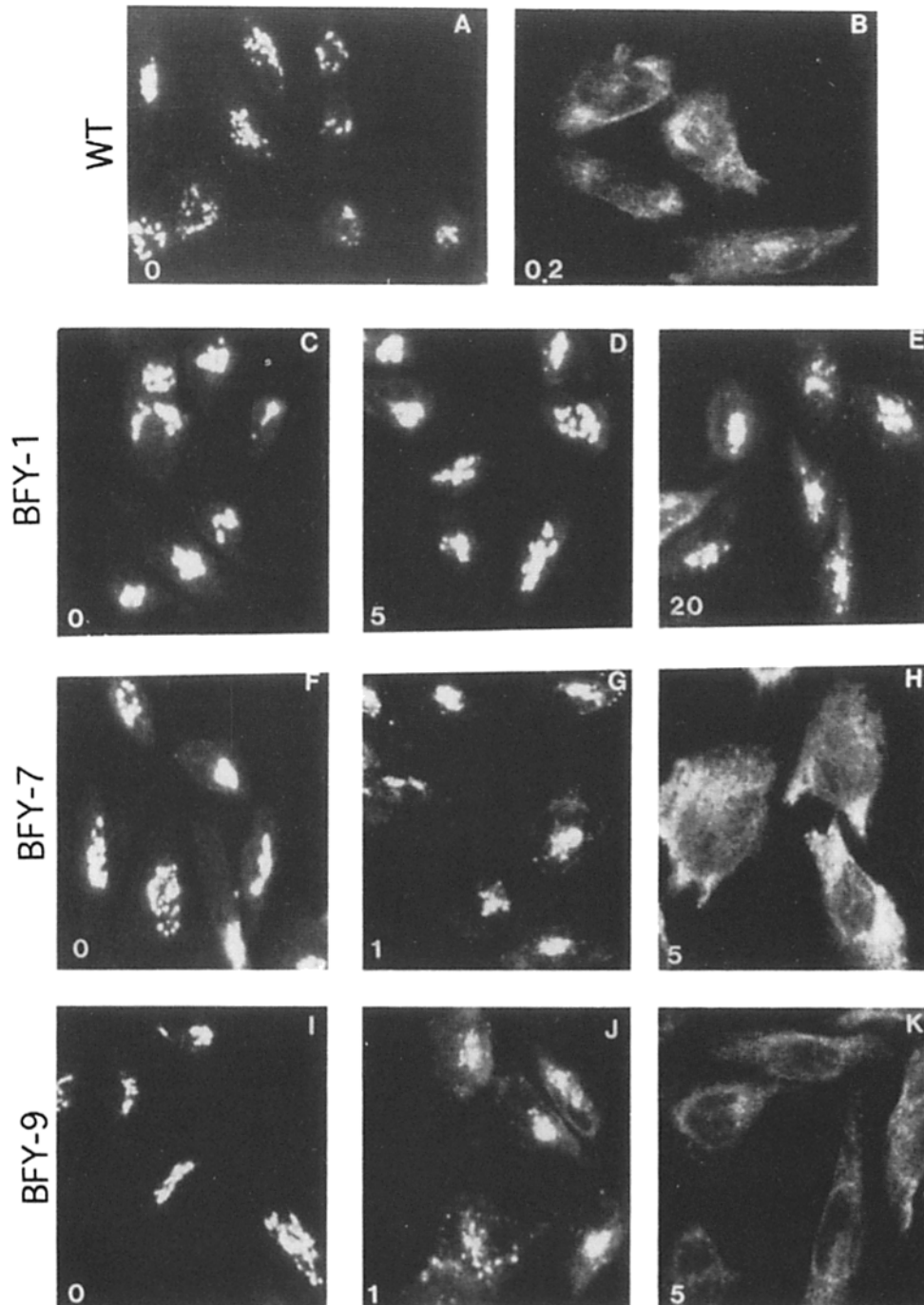The effect of BFA on the Golgi complex of mutant lines with

Figure 5. The Golgi morphology in CHO^BFY mutant lines is resistant to different levels of BFA. Wild-type (*A* and *B*), BFY-1 (*C-E*), BFY-7 (*F-H*), and BFY-9 (*I-K*) CHO cells were incubated in the absence and presence of various concentrations of BFA for 1 h and then washed. After fixation and permeabilization, the cells were incubated sequentially with a polyclonal antibody specific for Man II and a goat anti-rabbit IgG conjugated with rhodamine or FITC. The concentration of BFA used is given in the bottom left corner in units of μg/ml. The distribution of Man II is unaffected by BFA at 1 μg/ml in all mutant lines tested and at concentrations as high as 20 μg/ml in BFY-1 cells.
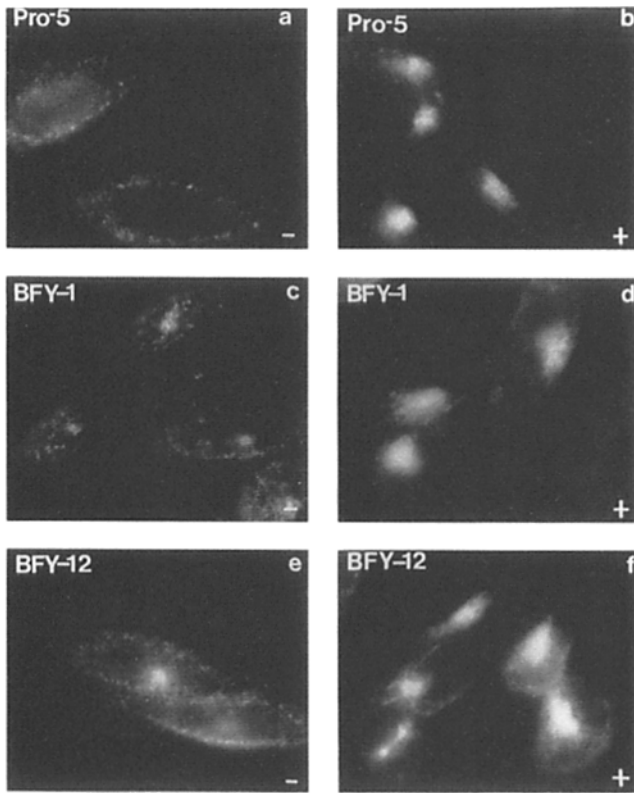
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Figure 6. BFA affects the distribution of early endosomes in all CHO[BFY] mutant lines tested. Early endosomes were labeled by incubating cells first with BSA (5 mg/ml) in α-MEM for 30 min, and then with 10 μg/ml of iron-saturated hTf for 1 h at 37°C. Cells were treated in the absence or presence (1 μg/ml) of BFA for 30 min at 37°C, followed by fixation with formaldehyde and permeabilization for immunofluorescence with a rabbit antibody to hTf and FITC-labeled goat anti–rabbit IgG. BFA causes redistribution of the early endosomes to a perinuclear location in all cells tested. A similar result was obtained even when using a lower concentration (0.2 μg/ml) of BFA.

the highest level of resistance to BFA was investigated by indirect immunofluorescence using an antibody to Man II, a Golgi marker. The results, presented in Fig. 5, demonstrate that while wild-type cells showed dispersed reticular staining in the presence of 0.2 μg/ml BFA, the appearance of the Golgi complex of BFY-1, -7, and -9 was not significantly changed at 1 μg/ml BFA.

However, at concentrations of BFA greater than 1 μg/ml, the Golgi morphology of mutant lines tested displayed different levels of resistance to BFA. Furthermore, the relative levels of resistance observed with this method were surprisingly different from the results obtained with the cell growth assay (see Fig. 1 and Table II). For example, whereas mutant lines BFY-1, -7, and -9 display similar levels of resistance in the growth assay (Table II), the distribution of Man II in BFY-7 and BFY-9 is completely sensitive to 5 μg/ml BFA (Fig. 5, *H* and *K*), but that in BFY-1 cells was resistant to BFA concentrations as high as 20 μg/ml (Fig. 5, *E*). A high level of resistance to BFA is not limited to the Golgi complex of BFY-1 cells. Indeed, Golgi morphology in three other cell lines (BFY-2, -3, and -12) remains normal at BFA concentrations as high as 10 μg/ml (data not shown). The observed differences in the level of resistance of Golgi morphology to BFA among mutants most likely result from mutations at different positions within a same gene, or in distinct genes. The lack of correlation between growth inhibition by BFA in mutant cells and the stability of Golgi morphology at high BFA concentration raises interesting questions about the mechanism of action of BFA which are addressed in more detail in the Discussion.

Early Endosomes of BFY Cells Are Sensitive to BFA

The effects of BFA are not limited to the Golgi apparatus. For example, treatment with BFA causes extensive tubulation and fusion of the early endosome and the TGN to form a hybrid organelle (Lippincott et al., 1991; Tooze and Hollinshead, 1992; Wood and Brown, 1992). Limited tubulation of lysosomes also results from BFA treatment in some cell types (Lippincott et al., 1991; Wood et al., 1991). For this reason, we determined what effect, if any, BFA had on these organelles in several of our BFY lines. Treatment with BFA at concentrations as high as 10 μg/ml for 1 h had no visible effect on the distribution of the two lysosome markers lgp95 and lgp58 (Joiner et al., 1990; van der Sluijs et al., 1992) in the wild-type CHO line (data not shown).

In contrast, a marked effect on the distribution of the early endosomes of wild-type CHO cells was observed upon treatment with 1 μg/ml BFA (Fig. 6, *a* and *b*). The clustering of the hybrid organelle resulting from the BFA-induced fusion of early endosomes with TGN has been best demonstrated using antibodies to TGN-38, a common marker of the TGN (Ladinsky and Howell, 1992). Several antibodies raised against rat TGN-38 (generous gifts of Dr. K. Howell,
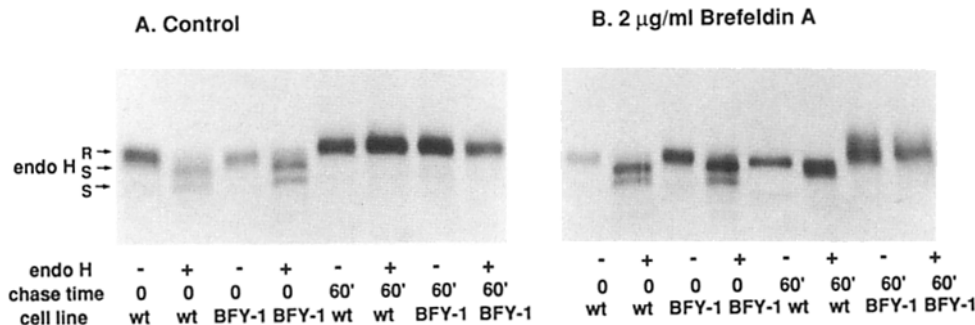
A. Control   B. 2 µg/ml Brefeldin A

Figure 7. Acquisition of endo-H resistance is not affected by BFA treatment in BFY-1 mutant cells. VSV-infected cells were pulse labeled for 15 min with [^{35}S]methionine and then chased for the indicated periods of time with cold methionine. Half of the cell samples were preincubated with 2 μg/ml BFA for 30 min before labeling and BFA levels were maintained during pulse and chase periods (*B*). After the chase, cell lysates were immunoprecipitated with a VSV-G antibody and one half of the immunocomplexes were then subjected to endo-H treatment, as indicated. The final results were visualized by SDS-PAGE. A fluorogram is shown.

University of Colorado Health Sciences Center, Denver, CO) were tested, but none were found to cross-react with CHO cells. As an alternative, EE were labeled by endocytosis of human transferrin, a marker used routinely for the identification of early endosomes (Hopkins, 1983; Hopkins and Trosbridge, 1983; Lippincott et al., 1991). The results shown in Fig. 6 demonstrate that the early endosome–TGN system of BFY-1 and BFY-12 cells have the same sensitivity to BFA displayed by the parent CHO wild-type cell line. This effect can be observed at a BFA concentration as low as 0.2 μg/ml. In all six mutant lines examined (BFY-1, -2, -3, -7, -12, and -21), BFA causes a clear redistribution of the endocytosed material to a perinuclear location under conditions where Golgi markers remain unaffected. This observation provides additional strong evidence for the presence of multiple organelle-specific targets for BFA. Finally, the sensitivity of EE/TGN of BFY mutant lines to low concentrations of BFA demonstrates that their BFA resistance cannot have arisen from the lack of import or increased export of the drug by a multi drug transporter.

Golgi Function Remains Normal in the Presence of BFA in BFY-1 Cells

The experiments in Fig. 5 established that the appearance of the Golgi in BFY-1 cells is unaffected by high concentrations of BFA and therefore suggest that Golgi function remains normal in this line despite the presence of the drug. Preliminary characterization of Golgi function in BFY-1 cells was performed by measuring total protein secretion from cells pulse labeled with [³⁵S]methionine. The results demonstrated that whereas secretion from the parent wild-type CHO line was completely inhibited by 1 μg/ml BFA during a 60-min chase period, secretion from BFY-1 cells remained normal at that concentration (data not shown). Golgi function in this line was investigated in more detail by measuring the effect of BFA on Golgi-dependent protein glycosylation in both mutant and wild-type cells, using resistance to endo-H as an assay. The loss of endo-H sensitivity indicates the conversion of asparagine-linked oligosaccharides from a high mannose to a complex form which occurs within the medial Golgi compartment.

In the experiment shown in Fig. 7, VSV-infected cells were pulse labeled with [³⁵S]methionine, and then chased for the indicated periods of time with cold methionine. As a control for the experiment, Fig. 7 A shows that the endo-H–sensitive forms of the VSV-G protein observed after the brief pulse were fully converted to endo-H–resistant forms during the 60-min chase in both wild-type and BFY-1 cells, in the absence of BFA. In contrast, a significant difference between wild-type and mutant cells was observed when cells were treated with 2 μg/ml BFA (Fig. 7, B). In wild-type CHO cells VSV-G was only partially resistant to endo-H after a 60-min chase, as previously reported (Doms et al., 1989). The partial processing of VSV-G most likely results from redistribution of Golgi enzymes to the ER (Doms et al., 1989). In contrast, the VSV-G protein made in mutant BFY-1 cells acquired endo-H resistance with kinetics similar to those observed in untreated cells. The abnormal migration of the VSV-G produced in BFY-1 cells in the presence of BFA could result from increased sialylation due to disruption of the endosome–TGN system (see Fig 6). We conclude that BFA causes a delay in the conversion of newly synthesized asparagine-linked

oligosaccharides from high mannose to complex forms in wild-type cells but has no such effect in mutant BFY-1 cells. The observation that the Golgi complex not only appears normal but also functions properly at a BFA concentration sufficient to inhibit growth (see Fig. 1) reveals a novel effect of BFA which is independent of its ability to disrupt organelle morphology.

Resistance to BFA in BFY-1 Cells Involves a Membrane-associated Factor Distinct from ARFs and Coatomers

One of the most immediate effects of BFA in wild-type cells is to interfere with assembly of protein coats on Golgi membranes (Klausner et al., 1992). This effect of BFA can be readily monitored as displacement of the 110-kD coat protein β-COP from Golgi membranes (Lipincott-Schwarz et al., 1989). Preliminary experiments using indirect immunofluorescence established that the membrane association of β-COP in BFY-1 cells was unaffected by BFA, even in semi-intact cells incubated with BFA-sensitive cytosol (data not shown). These results suggested that resistance to BFA resulted from a membrane-associated factor retained in the permeabilized cells. This possibility was investigated by preparing Golgi-enriched membrane fractions from either wild-type or mutant cells and determining their relative sensitivity to BFA using a cell-free assay that measures membrane association of coat proteins in the absence or presence of BFA (Fig. 8). In these assays, soluble components required for coat assembly were provided by cytosol prepared from BFA-sensitive CHO lec1 cells. We found that the extent of β-COP binding to wild-type and BFY-1 membranes in the absence of BFA was similar and corresponded to ∼25% of the total amount of β-COP present in the reaction. As shown in Fig. 8, association of β-COP–containing coatomers with wild-type Golgi membranes was inhibited by BFA in a dose-
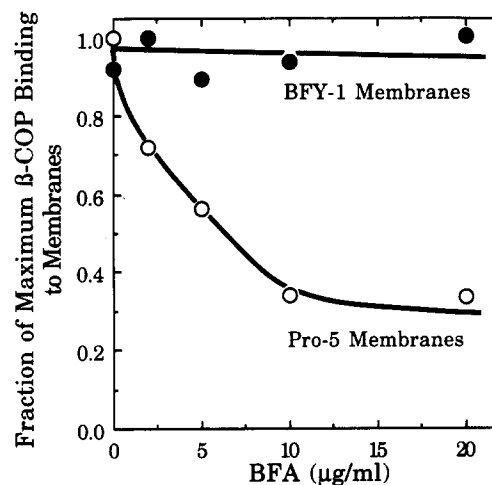

Figure 8. A BFA resistance factor is associated with Golgi membranes. Golgi-enriched membranes prepared either from wild-type (○) or from mutant BFY-1 (●) cells were incubated with cytosol prepared from BFA-sensitive CHO lec1 cells in the presence of the indicated BFA concentrations for 20 min at 30°C. The extent of β-COP associated with membranes at the end of the incubation was determined as described in Materials and Methods and is plotted as a function of BFA concentration. A phosphorImager print and a fluorogram are shown.

dependent manner. In contrast, association of coatomers with BFY-1 membranes was fully resistant to BFA. This observation demonstrates that the BFA resistance factor in BFY-1 cells is indeed membrane associated. Furthermore, the fact that similar amounts of β-COP bound to wild-type and BFY membranes suggests that resistance in BFY-1 cells does not result from a large increase in the affinity of coatomers for Golgi membranes.

Could ARFs or COPs be directly involved in the mechanism of resistance to BFA in BFY-1 cells? The observation that a BFA resistance factor is associated with the Golgi membranes of these cells makes this unlikely, since ARFs and COPs normally dissociate from membranes during isolation. Examination of the relative levels of ARF and β-COP in cell extracts verifies that wild-type and mutant membranes indeed carry equally low amounts of these coat proteins (Fig. 9). Quantitative analysis reveals that <10% of COPs in these binding assays are provided by membranes. Furthermore, the levels of ARF and coatomer in cytosol prepared from wild-type and mutant cells are also very similar (Fig. 9). This is in agreement with our observation that the levels of these proteins in total extracts of BFY-1 and BFY-12 cells are within experimental error (±5%) of those measured in wild-type cells. These results strongly suggest that resistance in these BFY lines does not result from mutations in ARFs or coatomers, or from changes in their expression level.

Discussion

The effects of the small fungal metabolite BFA on organelle morphology and function are dramatic: membranes tubulate and fuse into several separate subsystems from which protein exit is in some cases significantly altered. Much has been learned on the effects of BFA on several organelles and in a wide variety of cell types. However, the exact mechanism of action of BFA remains to be elucidated.

In this study, several CHO mutant cell lines (termed BFY) were isolated from EMS-mutagenized populations on the basis of their ability to grow in the presence of 1 μg/ml BFA. Extensive characterization of a subset of these mutant lines
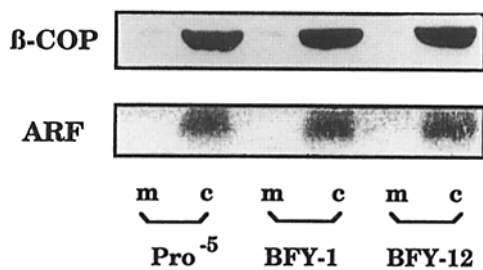
has established that most lines are genetically stable, and that BFA resistance does not result from trivial changes, such as enhanced inactivation by covalent modification or rapid efflux by a multidrug transporter. In addition, all mutant lines characterized were found to display organelle-specific resistance. Cell-free measurements with membrane extracts establish that resistance to BFA in BFY-1 cells involves a membrane-associated factor distinct from ARFs and coatomers. We conclude from these observations that resistance in these cells most likely arose from changes in the function of either an organelle-specific cellular target(s) for BFA or of a protein that interact with such a target(s).

Organelle-specific resistance to BFA has been reported in the two naturally resistant lines PtK-1 and MDCK (Hunziker et al., 1991; Ktistakis et al., 1991; Low et al., 1992; Apodaca et al., 1993). However, the relative effects of BFA on cell growth and organelle distribution in these lines were not investigated. The usefulness of these cell lines in identifying a cellular target(s) for BFA is further limited by the fact that resistance could have arisen over time from changes in several genes. Previous selections from mutagenized populations of Vero (Chen et al., 1992) and human carcinoma cells (Seguchi et al., 1992) have yielded three cell lines that can grow in presence of BFA. In all three cases, characterization revealed changes associated with the Golgi complex. However, the small number of mutants obtained in these studies and the lack of measurements on organelles other than the Golgi complex did not allow any generalizations about the mechanism of resistance to BFA. As described below, the collection and characterization of our CHO[BFY] lines overcomes these limitations and provides several novel insights.

A Multiple organelle-specific BFA targets
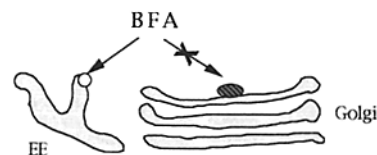
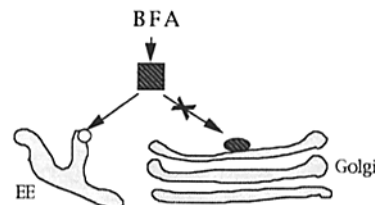B Multiple organelle-specific effectors for a single BFA target.

Figure 10. Two models for organelle-specific resistance to BFA in BFY lines. (*A*) EE and Golgi complex have distinct BFA targets. Mutation in the Golgi specific target can lead to BFA resistance. (*B*) A single BFA target acts on distinct organelle-specific effectors. Mutations in either the BFA target or Golgi-specific effector can lead to BFA resistance. If the mutation occurs in the BFA target, both EE and Golgi will be BFA resistant. In this model, the effector mutation could alter either the activity or the concentration of the effector in an organelle-specific manner.

β-COP

ARF

m c   m c   m c

Pro^{-5} BFY-1   BFY-12

Figure 9. BFA resistance does not arise from changes in the level or membrane association of ARFs and β-COP. Golgi-enriched membrane fractions (*m*) and cytosol (*c*) prepared from either pro^{-5}, BFY-1, or BFY-12 cells were subjected to SDS-PAGE. The amounts of membrane (2.5 μg) and cytosol (22.5 μg) loaded correspond to the ratios present in a COP-binding assay. Following transfer to nitrocellulose, blots were incubated with a β-COP specific antibody (m3A5) or [α-^{32}P]GTP to determine the relative levels of COPs and ARFs in those samples, as described in Materials and Methods. Quantitation of such blots establishes that greater than 90% of COPs and ARFs in all COP-binding assays are provided by cytosol.

BFA Action Involves Multiple Organelle-specific Proteins

Three separate selections yielded BFA-resistant mutants of CHO cells at a frequency of 2–30 per 10^7 mutagenized cells. The observation of such a high frequency in three separate attempts suggests that mutation of a single gene is sufficient to yield a BFA-resistance phenotype. Does this demonstrate that BFA has a single target in CHO cells? As discussed below, several of our observations indicate that this is not the case.

First, mutations conferring BFA-resistance in BFY cells do not eliminate all effects of BFA, but rather are organelle specific. Indeed, whereas the morphology of the Golgi complex in all BFY lines tested is completely unaffected by a 1-h treatment with 1 μg/ml BFA (or even 20 μg/ml in BFY-1 cells), the early endosomes in all six of the mutant lines characterized are disrupted by BFA in a manner identical to that observed in wild-type cells. It is particularly significant that the BFA sensitivities of these two organelles are nearly identical in the parent wild-type cells, but show large and varied differences in BFY lines. This dramatic change in the relative BFA sensitivities of the Golgi and early endosomes upon mutation in BFY cells can be best explained by the presence of multiple organelle-specific targets for BFA (Fig. 10, A), or by the presence of multiple organelle-specific "effectors" for the activity of a unique BFA target (Fig. 10, B). Model B predicts that some BFY lines could be obtained by mutation of the BFA target resulting in BFA resistance of both Golgi and endosomes. The availability of a large collection of mutant lines will allow a partial test of this prediction. Our observation to date that none of the six characterized BFY mutants show BFA resistance in both organelles favors A.

Second, whereas the growth of all BFY lines is clearly resistant to BFA relative to the wild-type parent line, none of those characterized grow above a BFA concentration of 2 μg/ml. In BFY-1 cells, this growth inhibition is not due to nonspecific effects such as disruption of the lipid bilayer by BFA (Zizi et al., 1991), since the inactive BFA analogues B27 and B36 cause no growth inhibition at concentrations as high as 3 μg/ml. This growth inhibition is not likely to result from known effects on organelles either, since Golgi function appears normal at BFA concentration as high as 2 μg/ml in BFY-1 cells. Rather, inhibition could result from the presence of an additional cellular target(s) for BFA which is affected only at high concentration and is not mutated in BFY cells. Elucidation of the mechanism causing growth inhibition in mutant cells may provide important new insights.

If BFA has multiple targets, how can BFA-resistant cells be obtained at such high frequency? Cell survival under our selection conditions require mutation only in target(s) significantly affected at 1 μg/ml BFA, and whose function was also essential. Our observation that all BFY lines tested have resistant Golgi morphology suggests that an intact Golgi complex is required for growth. In contrast, mutations leading to BFA-resistant endosomes were apparently not required. This can be in part explained by the fact that BFA does not block endocytosis (Lippincott et al., 1991; Wood and Brown, 1992). Furthermore, the ligand uptake and sorting carried out by the endosome–TGN system, which can be affected by BFA (Lippincott et al., 1991), may not be essential for growth under tissue culture conditions (Robbins et al., 1983; Klausner et al., 1984).

Identification of the Cellular Target(s) for BFA

Comparison of the effect of BFA on growth or the morphology of the Golgi complex in several BFY cells revealed extensive variation. Several explanations could account for these phenotypic variations. Mutations may have occurred at different positions within the same gene thereby reducing the affinity for the drug to different extents, or the level of expression of the wild-type or mutated BFA target could have been increased to different extents in the various mutant lines. Alternatively, future complementation analysis may show that mutations have been obtained in several different genes. Elucidation of the mechanism of resistance in the various lines could therefore lead not only to the identification of cellular targets for BFA, but also of proteins interacting with these targets. A whole cascade involved in regulating assembly of organelle-specific coats may thus be revealed.

Many biochemical assays can now be used to investigate the mechanism of resistance in BFY cells and identify components of this cascade. We have established that resistance to BFA in BFY-1 cells involves a membrane-associated factor which is distinct from ARFs and COPs. We conclude that resistance in these cells did not arise by overexpression of these downstream effectors of BFA, but more likely by mutation of the BFA target itself. For example, mutation may have occurred in the enzyme directly responsible for activation of ARFs by guanine nucleotide exchange, or in proteins regulating this exchange activity. Future measurements of the BFA effect on the ability of mutant Golgi-membranes to stimulate GTP-binding by ARFs (Donaldson et al., 1992; Helms and Rothman, 1992) will address these issues. In addition, since our results strongly suggest that resistance to BFA in BFY cells arose from a single mutation, cDNA expression cloning from BFY mutant libraries provides another very promising avenue for the identification of BFA target(s).

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