BFR1*, a Multicopy Suppressor of Brefeldin A-Induced Lethality, Is Implicated in Secretion and Nuclear Segregation in *Saccharomyces cerevisiae

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ABSTRACT

Brefeldin A (BFA) blocks protein transport out of the Golgi apparatus and causes disassembly of this organelle in mammalian cells. The primary effect of BFA is the release of the non-clathrin coat from Golgi membranes and vesicles. We sought to elucidate the mechanism of BFA action using a genetic approach in *Saccharomyces cerevisiae*. When an *erg6* *S. cerevisiae* strain is treated with BFA, cell growth is arrested, cells lose viability and secretory proteins are accumulated in the endoplasmic reticulum (ER) and early Golgi compartments. We demonstrate that the mutant *sec21* (defective in the *S. cerevisiae* homolog of γ -COP, a non-clathrin coat protein) is supersensitive to BFA. Hence BFA probably affects the same processes in *S. cerevisiae* as in mammalian cells. We used a multicopy genomic DNA library to search for multicopy suppressors of BFA-induced lethality. We identified one such gene, *BFR1*, that, in addition, partially suppresses the growth and secretion defects of the ER-to-Golgi secretion mutant *sec17*. A *bfr1*- $\Delta 1::URA3$ deletion strain is viable, but has defects in cell morphology and nuclear segregation, and the mutation accentuates the growth and secretion defects of a *sec21* mutant.

THE fungal toxin brefeldin A (BFA) dramatically alters the structures and functioning of the organelles of the secretory pathway (KLAUSNER *et al.* 1992; LIPPINCOTT-SCHWARTZ 1993). At the structural level, BFA causes the tubulation and eventual microtubule-dependent fusion of the Golgi apparatus with the endoplasmic reticulum (ER), the fusion of the trans-Golgi network (TGN) with endosomes, and the tubulation of the lysosome (HUNZIKER *et al.* 1992; KLAUSNER *et al.* 1992; PELHAM 1991). The effects of BFA on the ER and the Golgi have been the most extensively studied, and it has been demonstrated that BFA blocks anterograde vesicular traffic from the ER to the Golgi (KLAUSNER *et al.* 1992; LIPPINCOTT-SCHWARTZ 1993; MELANÇON *et al.* 1991). The earliest effect of BFA observed is the release of the peripheral Golgi membrane proteins β -COP and ARF (for ADP-ribosylation factor) from membranes into the cytoplasm (DONALDSON *et al.* 1990, 1991). This effect takes place within 30 sec after BFA addition and is therefore the first known molecular response to the drug. β -COP, one of the protein components of the non-clathrin coat found on ER and Golgi membranes and transport vesicles, cycles between membrane-bound and cytoplasmic forms (DUDEN *et al.* 1991; SERAFINI *et al.* 1991b; WATERS *et al.* 1991). ARF is a small GTP-binding protein involved in regulation of assembly of this coat onto membranes (DONALDSON *et al.* 1992a; SERAFINI *et al.* 1991a). Assembly of the non-clathrin coat onto membranes from the preformed cytosolic complex (referred to as the coatomer) is necessary for the formation of vesicles that transport proteins from the ER or one compart-

ment of the Golgi apparatus to the following compartment (MELANÇON *et al.* 1991; ORCI *et al.* 1991, 1993). It has recently been demonstrated that BFA inhibits the exchange of GTP for GDP onto ARF that is catalyzed by a Golgi membrane protein (DONALDSON *et al.* 1992b; HELMS and ROTHMAN 1992), but the molecular target(s) of BFA has (have) not yet been identified.

More than 20 genes have been identified that are involved in ER to Golgi transport in *Saccharomyces cerevisiae* (HARDWICK and PELHAM 1992; NAKAJIMA *et al.* 1991; NEWMAN and FERRO-NOVICK 1987; NEWMAN *et al.* 1990; OSSIG *et al.* 1991; PRYER *et al.* 1992). One group (including *SEC12*, *SEC13*, *SEC16*, *SEC23*, *SEC24* and *SAR1*) is necessary for the formation of ER-to-Golgi transport vesicles (BARLOWE *et al.* 1993; HICKE *et al.* 1992; KAISER and SCHEKMAN 1990; PRYER *et al.* 1993). *SAR1*, whose protein product is a small GTP-binding protein, was identified as a multicopy suppressor of *sec12* (NAKANO and MURAMATSU 1989). It has recently been shown that Sec12p is a GTP/GDP exchange protein for Sar1p (BARLOWE and SCHEKMAN 1993) and that Sec23p is a GTPase-activating protein for Sar1p (YOSHIIHISA *et al.* 1993).

SEC17 and *SEC18* encode the yeast homologs of the mammalian proteins, soluble NSF attachment protein (α -SNAP) and N-ethylmaleimide-sensitive factor (NSF), respectively, and are involved in vesicle targeting and/or fusion (CLARY *et al.* 1990; GRIFF *et al.* 1992; WILSON *et al.* 1989). NSF and α -SNAP have been demonstrated biochemically in mammalian *in vitro* systems to be necessary for fusion of ER-to-Golgi and

intra-Golgi transport vesicles with their acceptor compartments (BECKERS *et al.* 1989; WEIDMAN *et al.* 1989). α -SNAP is required for binding of NSF to membranes, and it in turn binds to a membrane-bound receptor (WILSON *et al.* 1992). Genetic evidence supports the idea that Sec17p and Sec18p also interact (KAISER and SCHEKMAN 1990). *BOS1*, *BET1/SLY12* and *SLY2/SEC22* encode homologs of the integral membrane protein synaptobrevin/VAMP, recently shown to be a vesicle-associated α -SNAP receptor (SÖLLNER *et al.* 1993). The *SED5* gene product is homologous to syntaxin, another α -SNAP receptor, but in this case associated with the acceptor compartment membrane (SÖLLNER *et al.* 1993). Although synaptobrevin/VAMP and syntaxin are specific to neural cells with functions limited to synaptic vesicle neurotransmitter release, the fact that yeast homologs have a demonstrated role in ER-to-Golgi transport *in vivo* provides evidence that this class of proteins has a general function in vesicle targeting and/or fusion.

A third group of genes involved in ER-to-Golgi and intra-Golgi transport includes *SEC21*, *SEC7*, *ARF1* and *ARF2*. The *SEC21* gene encodes the yeast homolog of γ -COP, a protein of the non-clathrin coat of ER-to-Golgi transport vesicles (HOSOBUCHI *et al.* 1992; STENBECK *et al.* 1992). *SEC7* encodes a protein associated with Golgi-derived vesicles, and Sec7p may be a vesicle coat protein as well (FRANZUSOFF *et al.* 1992; FRANZUSOFF and SCHEKMAN 1989; MELANÇON *et al.* 1991). *ARF1* and *ARF2* encode almost identical (and functionally interchangeable) proteins that are 74% identical to bovine ARF protein (STEARNS *et al.* 1990a,b). Synthetic lethal interactions among high temperature-sensitive (ts) mutants of *sec7* and *sec21* and the cold-sensitive (cs) *arf1* Δ mutant (STEARNS *et al.* 1990b) support the idea that the products of these genes act at a common step of ER-through-Golgi transport, perhaps the assembly/disassembly of the non-clathrin coat.

Although the effects of BFA on the structure and function of the ER and Golgi have been well characterized at a morphological and biochemical level in mammalian cells, the molecular mechanism of its action is unknown. In *S. cerevisiae*, several groups have recently shown that an *erg6* mutant is sensitive to BFA, and that secretory proteins are blocked in the ER and an early Golgi compartment in cells treated with BFA (GRAHAM *et al.* 1993; SHAH and KLAUSNER 1993; VOGEL *et al.* 1993). To identify proteins involved in the mechanism of action of BFA, we have undertaken a search for multicopy suppressors of the lethal effects of BFA in *S. cerevisiae*. We report the identification of a gene, *BFR1*, which in multiple copies partially suppresses the lethality and secretion defect caused by BFA and in addition partially suppresses the temperature sensitive growth and secretion defects of a *sec17* mutant. Disruption of the *BFR1* gene indicates that its protein product is implicated in normal nuclear

segregation and maintenance of cell size in addition to secretion.

MATERIALS AND METHODS

Strains, plasmids and materials: The *S. cerevisiae* strains used in this study are listed in Table 1. To construct the plasmid FKp132, the *Bam*HI-*Eco*RI fragment carrying the *erg6* Δ 1 gene [containing a deletion of the internal *Kpn*I fragment located within the *ERG6* gene (GABER *et al.* 1989)] was inserted into Yp352. To generate *erg6* Δ 1 strains, FKp132 was linearized with *Bst*XI, transformed into yeast strains, and *URA3* transformants selected. Mitotic recombinants that had lost the wild type *ERG6* gene were selected on plates containing fluoroorotic acid and tested for the *erg6* Δ 1 phenotype on a medium containing nystatin. The genomic DNA library carried in the plasmid pFL44L (BONNEAUD *et al.* 1991) was kindly provided by FRANÇOIS LACROUTE. Plasmids pCLJ10, pCLJ17, pCLJ101, pCLJ102 and pCLJ103 contain the portions of the insert shown in Figure 1 in the vector pFL44L. Plasmids pCLJ109, pCLJ110, pCLJ126 and pCLJ127 contain the portions of the insert shown in Figure 1 in the vector YEp352.

Media used and genetic methods performed are described in GUTHRIE and FINK (1991). DNA manipulations were performed as previously described (SAMBROOK *et al.* 1989). Nystatin was purchased from Sigma. BFA was purchased from Sigma and Epicenter Technologies, and was also obtained as a generous gift from Sandoz Laboratories.

DNA transformations were performed either by lithium acetate treatment (Ito *et al.* 1983) or by electroporation (MEILHOC *et al.* 1990) as described.

Selection of multicopy BFA suppressors: The strain R1992 *erg6* Δ 1 was transformed by electroporation with the genomic DNA library carried in the 2-micron vector pFL44L. Transformants were scraped off of selective plates into selective medium, and this culture was diluted to 10^5 cells/ml. Cells (2×10^5) from this culture were grown for two to three generations before addition of BFA to a final concentration of 100 μ g/ml and nystatin to a final concentration of 5 μ g/ml. Wild-type cells are sensitive to this concentration of nystatin, whereas an *erg6* Δ 1 mutant is resistant to up to 12 μ g/ml of this drug (data not shown). A preliminary experiment showed that, in the absence of nystatin, more than 99% of transformants obtained after treatment with BFA carried the wild-type *ERG6* gene. Hence nystatin was added to eliminate selection of plasmids carrying *ERG6*. After incubation for 12 hr, BFA and nystatin were added again; this treatment was repeated once more (but with only 2 μ g/ml nystatin), then cells were plated on YPD plates to recover surviving cells. Sixty-nine transformants were obtained, and plasmids were isolated from 44 of these yeast strains. All but three of these plasmids were represented only once, two were represented twice (including pCLJ10 and pCLJ17) and one plasmid was present four times. Among the multiply represented plasmids and 10 other plasmids isolated, only pCLJ10 and pCLJ17 reproducibly conferred resistance to BFA upon retransformation of R1992.

Radiolabeling and immunoprecipitation: Labeling of cells and immunoprecipitations were performed as previously described (D'ENFERT *et al.* 1991). Briefly, cells were grown overnight in minimal medium containing 100 μ M $(\text{NH}_4)_2\text{SO}_4$ to an OD_{600} of approximately 0.2 and centrifuged. Labeling was carried out in minimal medium without $(\text{NH}_4)_2\text{SO}_4$ at a cell concentration of 2 OD_{600} units. Labeling was performed with Trans³⁵S-label (purchased from ICN and distributed by Dositek, France), using 125–250 μ Ci per OD_{600} unit of cells, for either 10 or 15 min. A chase was carried out using non-radioactive cysteine and methionine at final concentrations of

TABLE 1

Strains used in this study

Strain	Genotype	Source
R1992	<i>MATα ura3-52 his4-15 lys9 erg6-Δ1</i>	GABER <i>et al.</i> (1989)
DBY877	<i>MATα his4-619</i>	ADAMS and BOTSTEIN (1989)
DBY2056	<i>MATα ura3-52</i>	D. BOTSTEIN
DBY2057 ^a	<i>MATα ura3-52</i>	ADAMS and BOTSTEIN (1989)
DBY5334	<i>MATα lys2-801 his3-Δ200 leu2-3,112 ura3-52 ypt1-1</i>	STEARNS <i>et al.</i> (1990b)
DBY5335	<i>MATα trp1-1 his3-Δ200 leu2-3,112 ura3-52 arf1::HIS3</i>	STEARNS <i>et al.</i> (1990b)
USO1-10	<i>MATα ura3-52 leu1 uso1-1</i>	NAKAJIMA <i>et al.</i> (1991)
RSY263 ^b	<i>MATα his4-619 ura3-52 sec12-4</i>	KAISER and SCHEKMAN (1990)
RSY265 ^b	<i>MATα his4-619 ura3-52 sec13-1</i>	KAISER and SCHEKMAN (1990)
RSY267 ^b	<i>MATα his4-619 ura3-52 sec16-2</i>	KAISER and SCHEKMAN (1990)
RSY269 ^b	<i>MATα his4-619 ura3-52 sec17-1</i>	KAISER and SCHEKMAN (1990)
RSY271 ^b	<i>MATα his4-619 ura3-52 sec18-1</i>	KAISER and SCHEKMAN (1990)
RSY275 ^b	<i>MATα his4-619 ura3-52 sec20-1</i>	KAISER and SCHEKMAN (1990)
RSY277 ^b	<i>MATα ura3-52 sec21-1</i>	KAISER and SCHEKMAN (1990)
RSY279 ^b	<i>MATα his4-619 ura3-52 sec22-3</i>	KAISER and SCHEKMAN (1990)
RSY281 ^b	<i>MATα his4-619 ura3-52 sec23-1</i>	KAISER and SCHEKMAN (1990)
CJY004	<i>MATα ura3-52 erg6-Δ1</i>	This study: <i>erg6-Δ1</i> in DBY2056
CJY001	<i>MATα his4-619 ura3-52 sec16-2 erg6-Δ1</i>	This study: <i>erg6-Δ1</i> in RSY268
CJY006	<i>MATα his4-619 ura3-52 sec17-1 erg6-Δ1</i>	This study: <i>erg6-Δ1</i> in RSY269
CJY002	<i>MATα his4-619 ura3-52 sec18-1 erg6-Δ1</i>	This study: <i>erg6-Δ1</i> in RSY271
CJY003	<i>MATα ura3-52 sec21-1 erg6-Δ1</i>	This study: <i>erg6-Δ1</i> in RSY277
MAY589	<i>MATα ade2-101 his3-Δ200 leu2-3,112 ura3-52</i>	A. HOYT
MAY591	<i>MATα lys2-801 his3-Δ200 leu2-3,112 ura3-52</i>	A. HOYT
CJY005	MAY589 $\times$ MAY591	This study
CJY021	DBY2057 $\times$ RSY269	This study
CJY031	CJY021 <i>bfr1-Δ1::URA3/BFR1</i>	This study
CJY031-8-3	<i>MATα ura3-52</i>	This study: segregant from sporulation of CJY031
CJY031-3-2	<i>MATα his4-619 ura3-52</i>	This study: segregant from sporulation of CJY031
CJY031-4-4	<i>MATα his4-619 ura3-52</i>	This study: segregant from sporulation of CJY031
CJY031-8-2	<i>MATα his4-619 ura3-52 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY031
CJY031-6-3	<i>MATα his4-619 ura3-52 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY031
CJY031-12-1	<i>MATα his4-619 ura3-52 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY031
CJY031-6-1	<i>MATα his4-619 ura3-52 sec17-1 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY031
CJY031-5-1	<i>MATα his4-619 ura3-52 sec17-1</i>	This study: segregant from sporulation of CJY031
CJY023	DBY2057 $\times$ RSY277	This study
CJY033	CJY023 <i>bfr1-Δ1::URA3/BFR1</i>	This study
CJY033-4-2	<i>MATα ura3-52 sec21-1 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY033
CJY033-5-1	<i>MATα ura3-52 sec21-1 bfr1-Δ1::URA3</i>	This study: segregant from sporulation of CJY033
CJY033-1-3	<i>MATα ura3-52 sec21-1</i>	This study: segregant from sporulation of CJY033
CJY033-2-1	<i>MATα ura3-52 sec21-1</i>	This study: segregant from sporulation of CJY033
CJY027	CJY031-6-3 $\times$ CJY031-8-2	This study
CJY028	CJY031-3-2 $\times$ CJY031-4-4	This study

^a Backcrossed ten times to DBY877.^b Backcrossed twice to DBY877 by CHRIS KAISER.

1 mg/ml, for either 20 or 30 min. Cells were lysed by agitation in the presence of glass beads, and immunoprecipitations carried out as described by D'ENFERT *et al.* (1991). Samples were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 7.5% acrylamide. Gels were treated with Amplify (purchased from Amersham), dried, and exposed to pre-flashed Kodak X-Omat K film for several days or weeks at -80° .

Cloning and sequence analysis of *BFR1*: Plasmids used to subclone the original plasmid carrying *BFR1* (pCLJ10) are shown in Figure 1. For technical reasons, we were unable to demonstrate that a wild-type copy of *BFR1* restores a wild-type phenotype to either the *bfr1- Δ 1::URA3* or *bfr1-2::URA3* strains. The single strain in which deletion of *BFR1* causes a phenotype carries only a *ura3-52* auxotrophic marker, and hence the *URA3* disruption strains have no requirements. Attempts to select *ura3* derivatives of the *URA3* disruption strains were not successful. However, the *bfr1-2::URA3* strain carries a simple insertion of *URA3* into the *BFR1* open reading frame (verified by Southern analysis), and hence it is very unlikely that the phenotypes of the *bfr1-2::URA3* strain are not due to disruption of the *BFR1* open reading frame.

DNA nucleotide sequences were determined by the dideoxy chain termination method as described previously (SANGER *et al.* 1977). Data bank searches were performed using the CITI2 computing facilities (DESSEN *et al.* 1990).

Microscopy: Yeast cells were grown to exponential phase in YPD medium, fixed in 70% ethanol for 30–60 min, washed in water, sonicated and resuspended in 1/10 volume of TE, pH 8, containing 0.5 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Preparations were viewed on a Zeiss Axio-phot microscope equipped for epifluorescence microscopy, and intact cells were visualized by phase contrast optics.

RESULTS

Brefeldin A arrests growth and reduces viability of an *erg6* mutant: Wild-type *S. cerevisiae* strains are resistant to BFA at concentrations up to 100 μ g/ml (data not shown) (GRAHAM *et al.* 1993; SHAH and KLAUSNER 1993; VOGEL *et al.* 1993). It has been reported that an *erg6* mutant, defective in the enzyme *S*-adenosyl-

FIGURE 1.—Restriction map of the region containing the *BFR1* gene and subcloning of the original plasmid carrying *BFR1*. The lines adjacent to each plasmid name indicate the portion of the genomic DNA insert contained in the plasmid. The deletion allele contained in plasmid pCLJ126, *bfr1-Δ1::URA3*, was constructed by replacing the region between the *Pst*I sites of pCLJ109 with the 1.1-kb *URA3-Hind*III fragment, and the insertion allele *bfr1-2::URA3* was obtained by inserting *URA3* into the unique *Bst*EII site within the *BFR1* coding region. Complementation analysis using suppression of the growth defect of strain RSY269 *sec17-1* was carried out at 33°, and that for BFA sensitivity is described in Table 2. ND indicates not determined.

methionine:Δ24-sterol-*C*-methyltransferase, which is necessary for ergosterol synthesis, is sensitive to a number of drugs, probably because of enhanced permeability of the plasma membrane (GABER *et al.* 1989; NITISS and WANG 1988; WINSOR *et al.* 1987). We found that an *erg6* mutant is also sensitive to BFA, as has recently been demonstrated by three other groups (data not shown, GRAHAM *et al.* 1993; SHAH and KLAUSNER 1993; VOGEL *et al.* 1993). After 8–10 hr of treatment with 100 μg/ml of BFA, cells are growth arrested and, in addition, only 10% of the cells remain viable (data not shown) (VOGEL *et al.* 1993). The fact that an *erg6-Δ1* strain is sensitive to BFA allows the selection of mutants or multicopy suppressors of the growth arrest and lethality caused by BFA.

***sec21* is supersensitive to BFA:** Since the first observed effect of BFA in mammalian cells is the release of β-COP (and presumably the entire non-clathrin coat) from membranes into the cytosol (KLAUSNER *et al.* 1992), we sought to determine the effect of BFA on a yeast mutant (*sec21*) defective in a subunit of the yeast non-clathrin coat complex (γ-COP) (HOSOBUCHI *et al.* 1992; STENBECK *et al.* 1992). Three other ER to Golgi mutants (*sec16*, *sec17* and *sec18*) were also tested for comparison. The *erg6-Δ1* disruption was introduced into each *sec* mutant strain to render the cells permeable to BFA.

The double mutant strains were grown to exponential phase and equal numbers of cells from each culture spotted onto YPD plates either with or without 60 μg/ml BFA and incubated at several different temperatures. In the absence of BFA, the maximum permissive temperature among those tested was 32° for *sec17* and *sec21*, and 26° for *sec16* and *sec18* (Figure 2). On the plates containing BFA, all strains including wild type were initially inhibited for growth, even at the permissive temperature of 24°. However, *sec17* and *sec18* eventually recovered and grew at their permissive temperatures as well as the *SEC* strain (Figure 2). *sec16* is the most temperature-sensitive of the mutants tested, being partially inhibited for growth even at 24°. At 24°, *sec16* eventually grew almost as well with BFA as without BFA (Figure 2). *sec21* on the other hand was severely inhibited for growth in the presence of BFA at the normally permissive temperature of 32° (Figure 2). Even at 24° and 26°, temperatures at which growth of *sec21* is normal in the absence of BFA, this mutant was clearly more inhibited for growth in the presence of BFA than *SEC*, *sec17* or *sec18* strains, and at 24° grew slightly less well than *sec16* (Figure 2). Hence *sec21* is more sensitive to BFA than *SEC*, *sec17* and *sec18* strains, and is probably more sensitive than *sec16* as well. These results support the hypothesis that the target of BFA in yeast acts at the same step of ER to Golgi transport as Sec21p, the yeast homolog of γ-COP.

It has been demonstrated recently that BFA blocks transport of secretory proteins and a vacuolar enzyme between the ER and the Golgi apparatus and in an early Golgi compartment (GRAHAM *et al.* 1993; SHAH and KLAUSNER 1993; VOGEL *et al.* 1993; our unpublished observations). These results, along with our genetic data linking the target of BFA to the yeast homolog of γ-COP, suggest that BFA has similar effects in *S. cerevisiae* as in mammalian cells.

Selection of multicopy suppressors of BFA sensitivity:

To identify the target of BFA itself or other interacting components, we selected multicopy suppressors of the lethal effects of BFA. Strain R1992 *erg6-Δ1* was transformed with a multicopy genomic DNA library carried in the 2-micron vector pFL44L (BONNEAUD *et al.* 1991). Twenty-two thousand transformants were obtained and subjected to BFA treatment. After 38 hr and three rounds of BFA addition, 69 viable colonies were recovered. Nystatin was added at 5 μg/ml (first two additions) or 2 μg/ml (last addition) to prevent growth of transformants carrying the *ERG6* wild-type gene (see MATERIALS AND METHODS). Such transformants were completely resistant to BFA as expected for a wild-type strain (data not shown). Plasmids from 44 of the 69 clones that survived the BFA selection were isolated and analyzed. Two were found to reproducibly confer resistance to BFA: pCLJ10 and pCLJ17. pCLJ17 carries a truncated version

FIGURE 2.—*sec21* is supersensitive to BFA. Cultures were grown to exponential phase and approximately equal quantities of cells spotted onto YPD plates with 60 μ g/ml or without BFA, and plates incubated at the indicated temperatures. Strains used were CJY001 *erg6- Δ 1 sec16*, CJY002 *erg6- Δ 1 sec18*, CJY003 *erg6- Δ 1 sec21*, CJY006 *erg6- Δ 1 sec17*, CJY004 *erg6- Δ 1 SEC+*.

of the insert found in pCLJ10 (Figure 1). The gene responsible for the suppression of BFA sensitivity carried on these plasmids will be called *BFR1* for *brefeldin A* resistance.

To quantify the level of resistance to BFA-induced lethality conferred by multicopy *BFR1*, cultures of strain R1992 *erg6- Δ 1* carrying the plasmid pCLJ10, pCLJ17 or the vector pFL44L without insert were treated with BFA to a final concentration of 100 μ g/ml. Cultures were incubated at 30° for 8–12 hr, then the same quantity of BFA was added again. After 12–16 hr incubation at 30°, the number of viable cells was determined. The percentage of cells originally present that remained viable after BFA treatment was 25-fold higher for cells carrying multicopy *BFR1* than for cells carrying the vector alone (Table 2).

To determine whether multicopy *BFR1* suppresses the growth arrest imposed by BFA, we determined growth rates of *erg6- Δ 1* strains carrying either pCLJ10 or the vector pFL44L at 30°. Growth of strains carrying either plasmid was not significantly slowed by treatment with 25 μ g/ml of BFA. However, at the twofold higher concentration of 50 μ g/ml BFA, the *erg6- Δ 1* strain was completely inhibited for growth. At this concentration of BFA, *erg6- Δ 1* cells carrying pCLJ10 did not grow significantly better than cells carrying the vector alone. We confirmed that pCLJ10 did suppress the lethality caused by BFA in this experiment, since we obtained 1×10^6 viable cells/ml for the *erg6- Δ 1*/pCLJ10 culture and only 3.75×10^3 cells/ml for the *erg6- Δ 1*/pFL44L culture at the 24 hr time point.

***BFR1* overexpression partially suppresses the temperature-sensitive growth defect of *sec17*:** To determine whether overexpression of *BFR1* suppresses defects in ER to Golgi transport other than that imposed

by BFA, we transformed a number of mutants defective in ER to Golgi transport with the plasmid pCLJ10 carrying *BFR1* or with the vector alone. Transformants of temperature-sensitive mutant strains *sec12*, *sec13*, *sec16*, *sec17*, *sec18*, *sec20*, *sec21*, *sec22*, *sec23* and *uso1* (Table 1) were incubated at 24°, 30°, 32° (or 33°), 36° and 37°. Transformants of the cold sensitive mutants *arf1* and *ypt1* (Table 1) were incubated at 16°, 24° and 30°. *sec17* was the only one of these mutants that showed a significant difference in growth between transformants carrying pCLJ10 and those carrying the vector pFL44L alone, at any of the semi- or nonpermissive temperatures tested (data not shown). The enhanced growth rate conferred on *sec17* by the plasmid pCLJ10 was seen at 33°, a temperature at which growth of *sec17* carrying the vector alone is clearly inhibited. To quantify the difference in growth rate conferred by multicopy *BFR1*, doubling times of strains carrying multicopy plasmids with or without *BFR1* were determined. *sec17* was transformed with pFL44L, pCLJ10, pCLJ109 (a subclone containing the entire *BFR1* gene, Figure 1) and pCLJ110 (a subclone in which the promoter and N-terminal quarter of the *BFR1* gene product is deleted, Figure 1). Transformants were grown at 25° in liquid culture to exponential phase. Cultures of each transformant were split and incubated either at 25° or 33°, and cell density monitored by determining the OD₆₀₀ of each culture at regular intervals. No difference in growth rates was observed at 25° for strains carrying plasmids with or without the intact *BFR1* gene (Table 3). At the semi-permissive temperature of 33°, cultures of *sec17* cells carrying the intact *BFR1* gene had doubling times of 1.7 and 1.8 hr, whereas cultures of cells carrying the vector alone or the truncated gene doubled their mass in 2.4 and 2.6 hr, respectively (Table 3).

TABLE 2
Multicopy *BFR1* confers resistance to BFA-induced lethality

Plasmid ^a	Description	Viability (%) ^b
pFL44L	Vector	0.032 ± 0.026 (9)
pCLJ10	Multicopy <i>BFR1</i>	0.79 ± 0.49 (10)
pCLJ17	N-terminal truncation of <i>BFR1</i>	0.16 ± 0.094 (5)
YEp352	Vector	0.035 ± 0.026 (6)
pCLJ109	Multicopy <i>BFR1</i> (Subclone of pCLJ10 in YEp352)	0.22 ± 0.057 (9)
pCLJ110	C-terminal truncation of <i>BFR1</i>	0.082 ± 0.04 (9)

^a Strain R1992 *erg6-Δ1* carrying either the plasmid pCLJ10 (four independent transformants), pCLJ17 (two independent transformants), or the vector pFL44L (two independent transformants), or the plasmids pCLJ109, pCLJ110 or the vector YEp352 (two independent transformants of each) were grown to exponential phase in selective medium. Cultures were diluted to 2×10^5 cells/ml, and BFA added to a final concentration of 100 μg/ml. Cultures were incubated at 30° for 8–12 hr, then the same quantity of BFA was added again. After 12–16 hr incubation at 30°, cells were plated onto YPD plates without BFA and colonies were counted 4 days later.

^b The mean and standard error of the number of independent determinations shown in brackets are given.

TABLE 3
Multicopy *BFR1* partially suppresses the temperature-sensitive growth defect of *sec17-1*

Strain	Doubling time (hr) ^a	
	25°	33°
RSY269 <i>sec17-1</i> /pFL44L	1.85	2.34
RSY269 <i>sec17-1</i> /pCLJ10	1.89	1.78
RSY269 <i>sec17-1</i> /pCLJ109	1.94	1.70
RSY269 <i>sec17-1</i> /pCLJ110	1.63	2.63

^a Cultures were grown overnight to exponential phase at 25°, split into two, and either left at 25° or shifted to 33°. The OD₆₀₀ of each culture was measured at regular intervals for 7.25 hr, and the doubling time calculated.

We also observed a very weak suppression of the growth defect of *sec21* by overexpression of *BFR1*. The effect was so slight that it was difficult to reproduce on plates, but in liquid culture the doubling time at 32° of a *sec21*/pFL44L strain was determined to be 2.6 hr and that of a *sec21*/pCLJ10 strain (carrying multicopy *BFR1*) was 2.2 hr. This marginal effect may be significant in light of the fact that a *bfr1-Δ1::URA3 sec21* double mutant is more temperature-sensitive and has a more pronounced secretion defect than *sec21* alone (see below).

Overexpression of *BFR1* partially suppresses the secretion defects imposed by BFA and *sec17*: To determine whether overexpression of *BFR1* suppresses the ER to Golgi secretion defects imposed by either BFA treatment or loss of *SEC17* function, we examined the fate of carboxypeptidase Y (CPY), a soluble vacuolar enzyme which traverses the ER and Golgi apparatus en route to the vacuole (STEVENS *et al.* 1982). Pro-CPY is core-glycosylated in the ER to produce the p1 precursor form (STEVENS *et al.* 1982). Addition of α1–6-linked mannose is limited, and produces a form of CPY that co-migrates with the ER-modified p1 form (FRANZUSOFF and SCHEKMAN 1989; GRAHAM *et al.* 1993). Addition of α1–2- and α1–3-linked mannose units produces the higher molecular weight p2 form, and removal of the pro region of CPY in the vacuole produces the lower molecular weight mature form (FRANZUSOFF and SCHEK-

MAN 1989; GRAHAM *et al.* 1993; STEVENS *et al.* 1982). Cells carrying multicopy *BFR1* or the vector alone were treated with 200 μg/ml of BFA for 40 min, then were labeled with a mixture of [³⁵S]methionine and [³⁵S]cysteine for 15 min (pulse) followed by the addition of cold methionine and cysteine for an additional 20 min (chase). Cell lysates were prepared from each sample, and were immunoprecipitated with anti-CPY antibodies. Without BFA treatment, cells carrying either the vector alone or multicopy *BFR1* accumulated exclusively the mature form of CPY after the 20-min chase period (Figure 3A, lanes 1, 2, 5 and 6). BFA-treated cells carrying the vector alone accumulated exclusively the p1 form of CPY, indicating a block in transport from the ER to the Golgi and from the early Golgi compartment in which α1–6-linked mannose is added, as described previously (Figure 3A, lanes 3 and 4). Cells carrying multicopy *BFR1* accumulated the same amount of the p1 precursor as cells carrying the vector alone, indicating that no suppression of the secretion defect imposed by BFA occurs at this high concentration of BFA (Figure 3A, lanes 7 and 8). The same result was obtained for cells treated with 50 μg/ml BFA, a concentration that severely inhibits transport of CPY (Figure 3B). In cells treated with only 25 μg/ml BFA, a significant amount of the mature vacuolar form of CPY was observed, indicating that transport from the ER to the Golgi was not completely blocked, but the abnormally high level of the p1 precursor indicates that transport from the ER was slowed at this concentration of BFA (Figure 3B). Cells carrying multicopy *BFR1* showed less accumulation of the p1 precursor and more of the mature vacuolar form of CPY than cells carrying the vector alone at 25 μg/ml BFA (Figure 3B). Hence multicopy *BFR1* partially suppresses the partial block to CPY transport imposed by a low concentration of BFA.

sec17 cells carrying either pCLJ10 or pFL44L, and a wild-type strain, were incubated at 26° or 34° for 1 hr prior to pulse-chase analysis as described above. At 34°, a nonpermissive temperature for *sec17*, cells carrying either pCLJ10 or pFL44L accumulated primarily the p1

FIGURE 3.—Multicopy *BFR1* partially suppresses the secretion blocks imposed by BFA and *sec17*. (A) Strain R1992 *erg6-Δ1* carrying either pCLJ10 (containing the *BFR1* gene) or pFL44L (the vector alone) were pretreated for 40 minutes at 30° with BFA at a final concentration of 200 μg/ml (lanes 3, 4, 7 and 8), or without BFA (lanes 1, 2, 5 and 6). Cultures were then radiolabeled for 10 min with ³⁵S, chased for 20 min, cell lysates prepared and immunoprecipitations with anti-CPY antibodies carried out. Samples taken either immediately after the labeling period (0-min chase) or after the 20-min chase period (20 min chase) were then subjected to SDS-polyacrylamide gel electrophoresis and fluorography. (B) Pulse-chase experiments were carried out as described in part A, after pretreating cultures for 30 min with 0, 25, 50 or 200 μg/ml BFA at 30°. Only samples obtained after the 20-min chase period are shown. (C) Cultures of strain RSY269 *sec17-1* carrying either pFL44L or pCLJ10 (multicopy *BFR1*) were grown overnight at 22°, then shifted to 26° for 30 min prior to pulse-chase analysis. ³⁵S-labeling was carried out for 15 min, followed by a 20-min chase period.

form of CPY (data not shown). Hence at this temperature there was no observable suppression of the secretion defect of *sec17* by multicopy *BFR1*. At 26°, a significant amount of the p1 precursor accumulated in the *sec17* mutant strain as compared to the wild-type strain, indicating a partial secretion defect even at this permissive temperature for growth (Figure 3C). *sec17* cells carrying multicopy *BFR1* accumulated less p1 precursor than cells carrying the vector alone, indicating a partial suppression of the *sec17* secretion defect at the semi-permissive temperature of 26° (Figure 3C).

***BFR1* is not an essential gene, but *bfr1-Δ1::URA3* cells are larger than normal:** To determine whether *BFR1* is necessary for vegetative growth, two gene deletions were constructed. In the first, the 1.5 kilobase (kb) *Pst*I fragment of pCLJ109 (Figure 1) was replaced with a 1.1-kb fragment carrying the selectable marker *URA3*. In the resulting construct, *bfr1-Δ1::URA3*, 77% of the *BFR1* gene is deleted, leaving only 325 (out of 1412) base pairs of the 5-prime end of the gene. In the second, the *URA3* gene was inserted into the unique *Bst*EII site within the *BFR1* coding region to generate *bfr1-2::URA3* (Figure 1). A *MATa*/*MATα* diploid strain homozygous for *ura3-52* (CJY005) was transformed with a fragment carrying the *bfr1-Δ1::URA3* gene, transformants were verified by Southern blotting, and heterozygous *BFR1*/*bfr1-Δ1::URA3* diploids sporulated. Tetrads were dissected and all four spores of each tetrad grew into identically sized colonies at 30°. The *bfr1-Δ1::URA3* cells appeared identical to wild-type cells, and were neither ts nor cs. However, when this experiment was repeated using a different diploid strain (CJY031), examination of cells from *bfr1-Δ1::URA3* colonies revealed that they were considerably larger than normal haploid cells (Figure 4). This large cell phenotype cosegregated 100% with the *URA3* gene marking the *bfr1-Δ1::URA3* disruption in 28 tetrads. Since the *bfr1-Δ1::URA3* allele deletes sequences downstream of the *BFR1* gene, we repeated this experiment using the *bfr1-2::URA3* insertion allele, which inactivates only the *BFR1* open reading frame. Sporulation of the *BFR1*/*bfr1-2::URA3* diploid strain and dissection of tetrads generated results identical to those for the *BFR1*/*bfr1-Δ1::URA3* diploid strain; the large cell phenotype segregated 2:2 and was linked 100% with uracil prototrophy. Hence this phenotype is due to inactivation of the *BFR1* open reading frame.

The *bfr1-Δ1::URA3* mutation did not significantly inhibit growth of a haploid strain, since the doubling time at 30° of CJY031-6-3 *bfr1-Δ1::URA3*, was 1.4 hr whereas the doubling time of a wild-type segregant, CJY031-8-3, was 1.3 hr. Interestingly, *bfr1-Δ1::URA3*/*bfr1-Δ1::URA3* *MATa*/*MATα* diploids grew considerably more slowly than *BFR1*/*BFR1* *MATa*/*MATα* diploids. *bfr1-Δ1::URA3*/*bfr1-Δ1::URA3* *MATa*/*MATα* diploids also exhibited more pronounced phenotypes than *bfr1-Δ1::URA3* *MATa* or *MATα* haploids (see below).

We tested whether deletion of *BFR1* conferred a supersensitivity or resistance to BFA. Isogenic *erg6-Δ1* and *erg6-Δ1 bfr1-Δ1::URA3* strains were constructed and treated with 200 μg/ml BFA for 24 hr. The two *erg6-Δ1* strains tested had 0.27 and 1.1% viable cells after this treatment, compared with 0.6 and 1.02% viable cells for two *bfr1-Δ1::URA3 erg6-Δ1* strains. Hence deletion of *BFR1* does not affect the sensitivity of an *erg6-Δ1* strain to BFA.

FIGURE 4.—Cellular and nuclear morphologies of *bfr1-Δ1::URA3* haploid and *bfr1-Δ1::URA3/bfr1-Δ1::URA3* diploid cells. Strains were grown to exponential phase, fixed, and stained with DAPI to visualize DNA (see MATERIALS AND METHODS). (A) CjY031-8-3 *BFR1*, (B) CjY031-6-3 *bfr1-Δ1::URA3*; (C) CjY028 *BFR1/BFR1*; (D) CjY027 *bfr1-Δ1::URA3/bfr1-Δ1::URA3*. Scale bar in part A is approximately 5 μ m.

***bfr1-Δ1::URA3* cells are defective in nuclear segregation:** Often a large cell phenotype is associated with a defect in the cell cycle (PRINGLE and HARTWELL 1981). To determine whether *bfr1-Δ1::URA3* cells accumulated at a particular stage of the cell cycle, we examined the distribution of unbudded, small budded and large budded cells in *BFR1* and *bfr1-Δ1::URA3* strains. Strain CjY031-8-3 *BFR1* had 32% unbudded, 36% small budded and 31% large budded cells, whereas *bfr1-Δ1::URA3* strains CjY031-6-3 and CjY031-12-1 (results pooled) had 29% unbudded, 37% small budded and 34% large budded cells. These results show that there is no significant difference between the distribution of cells at different stages of the cell cycle in *bfr1-Δ1::URA3* and wild-type strains, and hence that the large cell phenotype is not due to a delay in traversing a particular point in the cell cycle.

To determine their nuclear phenotypes, *bfr1-Δ1::URA3* and wild-type haploid cells were stained with DAPI and examined microscopically. The *bfr1-Δ1::URA3* strain clearly had an elevated level of abnormal nuclear phenotypes: 2.7% of these cells had no nucleus, and 1.1% had two nuclei in one cell body (Figure 4, Table 4). In addition to having a homogeneous population of large but nevertheless normally shaped cells, cultures of the *bfr1-Δ1::URA3* mutant also had a low percentage of cells with an abnormal morphology (see Figure 4 for examples). Abnormal morphologies included very elongated cells, cells with an irregular shape and cells in chains indicative of a defect in cytokinesis. Essentially all of these abnormally shaped cells exhibited abnormal nuclear phenotypes. The *bfr1-Δ1::URA3/bfr1-Δ1::URA3* *MATa/MATα* diploid

strain had a more significant nuclear segregation defect than the *bfr1-Δ1::URA3* *MATa* or *MATα* haploid strains. Of the *bfr1-Δ1::URA3/bfr1-Δ1::URA3* *MATa/MATα* cells, 7.7% were anucleate and 3.5% had two nuclei in one cell body. Of these cells, 2.7% had an abnormal morphology and associated nuclear abnormalities (Figure 4, Table 4). Hence *bfr1-Δ1::URA3* mutants are defective in nuclear segregation.

***bfr1-Δ1::URA3* enhances the secretion defect of *sec21*:** To determine whether a *bfr1-Δ1::URA3* strain is defective in secretion, isogenic *bfr1-Δ1::URA3* and *BFR1* strains were pulse-labeled for 10 min and chased for 20 min. Cell lysates were immunoprecipitated sequentially with anti-CPY and then with anti- α -factor antibodies as described above. There was no detectable difference in accumulation of intracellular forms of CPY or of α -factor between the *bfr1-Δ1::URA3* and *BFR1* strains (data not shown). Hence a *bfr1-Δ1::URA3* strain is not defective in intracellular transport.

We investigated whether *bfr1-Δ1::URA3* affects the temperature-sensitive growth defects of *sec17*, *sec18* and *sec21* mutant strains. Double mutant *sec bfr1-Δ1::URA3* strains were constructed and incubated at 24°, 30°, 33° and 36°. The *bfr1-Δ1::URA3* mutation neither suppressed nor accentuated the growth defects of *sec17* or *sec18* at any of these temperatures. However, *sec21 bfr1-Δ1::URA3* strains grew considerably more slowly at the semipermissive temperature of 33° than *sec21* strains (Figure 5A). Hence *bfr1-Δ1::URA3* accentuates the temperature-sensitive growth defect of a *sec21* strain.

The effect of the *bfr1-Δ1::URA3* mutation on the secretion defects of *sec17* and *sec21* mutant strains was tested. Wild-type, *sec17*, *sec17 bfr1-Δ1::URA3*, *sec21*

TABLE 4
Abnormal nuclear phenotypes of *bfr1-Δ1::URA3* strains

Phenotype scored	<i>bfr1-Δ1::URA3</i> ^a		<i>BFR1</i>		<i>bfr1-Δ1::URA3</i> / <i>bfr1-Δ1::URA3</i>		<i>BFR1/BFR1</i>	
	No. of cells	%	No. of cells	%	No. of cells	%	No. of cells	%
Anucleate cells	43	2.7	1	0.06	89	7.7	4	0.18
Two nuclei in one cell body	17	1.1	3	0.17	41	3.5	9	0.4
Three or more cell bodies, abnormal nuclei ^b	9	0.6	0	<0.06	31	2.7	0	<0.04
Totals	69	4.4	4	0.23	161	13.9	13	0.58
Total cells counted	1594		798		1157		2258	

^a Strains used were CJY021-6-3 *bfr1-Δ1::URA3*, CJY021-8-3 *BFR1*, CJY027 *bfr1-Δ1::URA3/bfr1-Δ1::URA3* and CJY028 *BFR1/BFR1*.

^b Either one or more anucleate cell bodies, a cell body with multiple nuclei, or both.

FIGURE 5.—Augmentation of the growth and secretion defects of *sec21* by *bfr1-Δ1::URA3*. (A) Strains CJY033-4-2 *sec21 bfr1-Δ1::URA3* and CJY033-2-1 *sec21 BFR1* were grown to exponential phase, and approximately equal quantities (normalized according to OD₆₀₀) were spotted onto YPD plates, and incubated at either 24° or 33° for 2 days. A fivefold higher concentration of cells was used in the first and third columns as in the second and fourth columns. (B) Strains CJY033-5-1 *sec21bfr1-Δ1::URA3*, CJY033-1-3 *sec21BFR1* and CJY031-8-3 *SEC21 BFR1* were preincubated for 45 min at 33°, ³⁵S-labeled for 15 min, then chased for 30 min. Samples were removed directly after the pulse and after the chase period, and processed as described in Figure 5 using anti-CPY antibodies.

and *sec21 bfr1-Δ1::URA3* strains were preincubated for 45 min at the semipermissive temperatures of 34° (for the *sec17* and wild-type strains) or 33° (for the *sec21* strains), then subjected to pulse-chase analysis and immunoprecipitation with antibodies against CPY as described above. *sec17* accumulated more of the p1 form of CPY than a wild-type strain, indicating a partial ER-to-Golgi transport defect of the *sec17* mutant at 34°, but the presence of the mature form indicated that transport was not completely blocked at this step. Exactly the same result was observed for the *bfr1-Δ1::URA3 sec17* double mutant strain, indicating that *bfr1-Δ1::URA3* does not affect the partial ER-to-Golgi transport defect of *sec17* at 34° (data not shown). The *sec21* mutant at 33° exhibits an enhanced accumulation of the p2 form of CPY (but not of the p1 form) after the ten-minute pulse as compared with the wild-type strain, but after 30

min of chase only the mature form is visible (Figure 5B). Hence the *sec21* mutant has only a partial secretion defect at 33°. Interestingly, this defect is at a later stage in protein transport than is observed at the completely restrictive temperature of 37°, at which *sec21* accumulates exclusively the p1 form of CPY (present in the ER and perhaps an early Golgi compartment) (HOSOBUCHI *et al.* 1992). The *sec21 bfr1-Δ1::URA3* double mutant strain accumulated significantly more of the p2 form than the *sec21* single mutant strain, indicating that the *bfr1-Δ1::URA3* mutation accentuates the late-Golgi transport defect of *sec21* (Figure 5B).

Sequence of *BFR1*: Subcloning of the original plasmid carrying the *BFR1* gene narrowed the region conferring BFA resistance and suppression of *sec17* to a 3-kb *SphI* fragment (Figure 1; see also Table 2). A 2.2-kb portion of this region was sequenced. One large open reading frame was found, with the potential to encode a protein of 470 amino acids (Figure 6A). The KYTE-DOOLITTLE (1982) hydropathy plot for the putative *BFR1* protein product (Bfr1p) indicates that the protein is very hydrophilic, with no potential membrane-spanning domains (Figure 6B). The predicted Bfr1p protein is predicted to possess 38.3% charged residues. Since 99% of yeast proteins have a frequency of charged amino acids that does not exceed 36.7% (KARLIN and BRENNEL 1992), this result indicates that Bfr1p is one of the most highly charged proteins of *S. cerevisiae*.

Sequencing of an adjacent region revealed that the *BFR1* gene is located 1.8 kb from the gene *HIS3* and adjacent to *PET56*, on the right arm of chromosome XV (Figure 1). Genetic analysis also indicated that *BFR1* is tightly linked to *HIS3* (data not shown).

The predicted Bfr1p sequence was compared to existing amino acid sequences in the NBRF and SWISS-PROT data banks using the FASTA program (PEARSON 1990). No sequences with significant homology to Bfr1p were found. Weak homologies (14–17% identities) with a number of proteins capable of forming a coiled-coil were observed. This type of secondary structure is characterized by the ability to form an α -helix, and by heptad repeats in which the first and fourth amino acids are

FIGURE 6.—(A) Nucleotide and predicted amino acid sequences of the *BFR1* gene. Numbers to the right and left of each line of DNA sequence refer to the nucleotide position, and numbers adjacent to the protein sequence refer to the amino acid position. The sequence reported here has been deposited in the GenBank data base (accession no. U06870). (B) KYTE-DOOLITTLE (1982) hydropathy plot of the protein sequence encoded by *BFR1* [as performed by DNA STRIDER (MARCK 1988)]. (C) Prediction of coiled-coil-containing regions of the Bfr1p protein sequence, using the program of LUPAS *et al.* (1991).

enriched in hydrophobic residues. There are no extensive regions within Bfr1p that possess these characteristics, but three small regions of the protein sequence (from 69 to 80 amino acids long) are predicted to form a coiled-coil (LUPAS *et al.* 1991) (Figure 6C).

DISCUSSION

Selection of multicopy suppressors of BFA-induced lethality: The drug BFA has been shown in mammalian cells to disrupt the structures of the ER, Golgi, TGN, endosomes and lysosome, and to inhibit certain protein trafficking steps (HENDRICKS *et al.* 1992b; HUNZIKER *et al.* 1992; KLAUSNER *et al.* 1992; LADINSKY and HOWELL 1992; MILLER *et al.* 1992; REAVES and BANTING 1992; ROSA *et al.* 1992; TOOZE and HOLLINSHEAD 1992; WOOD and BROWN 1992). The effects of BFA on the ER and the Golgi were the first identified and have been the most thoroughly studied at the molecular level (KLAUSNER *et al.* 1992; LIPPINCOTT-SCHWARTZ 1993). In this system, the primary effect of the drug is to prevent assembly onto Golgi membranes of the non-clathrin coat necessary for vesicle formation and therefore to block anterograde protein transport to the Golgi apparatus (DUDEN *et al.* 1991; HENDRICKS *et al.* 1993; MELANCON *et al.* 1991; OPRINS *et al.* 1993; ORCI *et al.* 1991; SERAFINI *et al.* 1991b; WATERS *et al.* 1991). Retrograde transport from the Golgi to the ER, which is dependent on the presence of intact microtubules, continues in the presence of BFA, which eventually results in the disassembly of the Golgi apparatus (DOMS 1989; LIPPINCOTT-SCHWARTZ *et al.* 1990). In some (but not all) cell types, proteins and membrane normally localized to the Golgi are redistributed to the ER in the presence of BFA (DE LEMOS-CHIARANDINI *et al.* 1992; HENDRICKS *et al.* 1992a; HIDALGO *et al.* 1992; HURTLEY 1992; ULMER and PALADE 1991). Although there is some heterogeneity among different cell types as to the extent of Golgi disassembly, in every case so far examined, BFA disrupts Golgi structure. Our results and those of others (GRAHAM *et al.* 1993; SHAH and KLAUSNER 1993; VOGEL *et al.* 1993) indicate that BFA inhibits ER-to-Golgi and intra-Golgi transport in *S. cerevisiae* as it does in mammalian cells. The secreted proteins α -factor and invertase, and the vacuolar protein CPY are accumulated intracellularly in partially glycosylated forms indicative of an inhibition of transport from the ER to the Golgi and through early Golgi compartments. We observed, in addition, that a *sec21* mutant is supersensitive to BFA. Since *SEC21* encodes the *S. cerevisiae* homolog of the non-clathrin coat protein γ -COP (HOSOBUCHI *et al.* 1992; STENBECK *et al.* 1992), this result is consistent with the idea that BFA affects the functioning of the non-clathrin coat in yeast. We conclude that BFA inhibits an important ER through Golgi transport mechanism in both yeast and mammalian cells, and therefore that yeast provides a good model system for identifying the protein components involved in this mechanism.

Our goal was to identify the target of BFA and other proteins implicated in the protein transport step(s) disrupted by BFA. We chose the approach of multicopy suppression of BFA-induced lethality to achieve this goal for several reasons. First, this approach has been used successfully to identify the targets of three drugs: tunicamycin, compactin and ethionine (RINE *et al.* 1983). Second, multicopy suppressors (usually of the lethality induced by a ts mutation but also valid for a drug-induced lethality) are often genes encoding components of a protein complex (CHIANNILKULCHAI *et al.* 1992; HADWIGER *et al.* 1989; SURANA *et al.* 1991) or proteins implicated in the same regulatory pathway (MACKAY 1983; BENDER and PRINGLE 1989). Third, identification of the gene responsible for the multicopy suppression is simplified by the fact that the gene is already cloned. We transformed an *erg6* strain sensitive to BFA with a genomic DNA library carried in a multicopy vector, and selected transformants resistant to BFA-induced lethality. One gene was identified, *BFR1*, that conferred a reproducible resistance to the lethal effects of BFA in multiple copies. Multicopy *BFR1* suppressed partially the lethality and secretion defects induced by BFA treatment, but did not suppress the growth defect imposed by BFA, even after 24 hr of treatment with the drug. In the simplest model, overexpression of the target of a drug should confer a suppression of the growth inhibition of the drug, as is indeed the case for the target of tunicamycin (RINE *et al.* 1983). However, we found no suppressors of BFA-induced growth inhibition in our selection. This result may indicate that the target of BFA is part of a complex and that overexpression produces only the monomeric form of the protein [as has been demonstrated for the products of the secretion genes *SEC13* and *SEC23* (HICKE *et al.* 1992; PRYER *et al.* 1993)]. If the oligomeric form of the target protein were uniquely sensitive to the drug, little or no suppression would be seen by overexpression of the protein. Another possible explanation is that overexpression of the target of BFA is detrimental to the cell. It is interesting to note that overexpression of the small GTP-binding protein ARF in yeast confers a severe growth inhibition (STEARNS *et al.* 1990a), and that a target of BFA in mammalian cells is implicated in the activation of a mammalian homolog of ARF (DONALDSON *et al.* 1992b; HELMS and ROTHMAN 1992). We can conclude that the product of the *BFR1* gene is not the sole target of BFA in *S. cerevisiae*, since deletion of the gene does not restore resistance to BFA (see NITISS and WANG 1988). It remains possible that there is a second gene encoding a protein with an overlapping function which is also inactivated by BFA.

The deduced sequence of the protein encoded by the *BFR1* gene (Bfr1p) does not provide much information as to its function. No similar sequences were found in the

protein data banks. Bfr1p is predicted to have a molecular weight of approximately 55 kD and to be very hydrophilic. Since the ratio of charged to apolar residues is high, Bfr1p is predicted to adopt a rod-shaped rather than a globular configuration (COHEN and PARRY 1986). Indeed, a large portion of Bfr1p is predicted to be α -helical by the Chou-Fasman and Garnier-Robson-Gibrat programs (data not shown). Three short regions of the protein are predicted to form a coiled-coil based on the algorithm of LUPAS *et al.* (1991). Several proteins involved in secretion also have short regions predicted to form a coiled-coil, including the *SEC2* and *SED5* gene products (NAIR *et al.* 1990; PELHAM 1993). The coiled-coil region of Sec2p is essential for its function in protein transport, and it has been proposed that the region may be involved in protein-protein interactions (NAIR *et al.* 1990). Determination of whether Bfr1p is part of a protein complex and identification of potential interacting proteins will await production of antibodies.

***BFR1* is implicated in nuclear segregation:** Disruption of the *BFR1* gene indicates that it is not essential for vegetative growth of yeast cells. However, there are several examples in yeast of duplicated genes or of genes with overlapping functions (SCHATZ *et al.* 1986a,b; STEARNS *et al.* 1990a; BENDER and PRINGLE 1991). We have not excluded the possibility that a redundant function exists for Bfr1p. Although *bfr1-Δ1::URA3* deletion strains are viable, they have several interesting phenotypes; 3.8% of *bfr1-Δ1::URA3 MATa* or *MATα* haploid cells and 11.2% of *bfr1-Δ1::URA3/bfr1-Δ1::URA3 MATa/MATα* diploid cells are either anucleate or have two nuclei in one cell body, indicating a defect in nuclear positioning or segregation during mitosis. Proper positioning of the nucleus during mitosis is mediated by three cytoskeletal systems in yeast: the cytoplasmic microtubules, the actin cytoskeleton and the intermediate filament network (HUFFAKER *et al.* 1987, 1988; JACOBS *et al.* 1988; PALMER *et al.* 1992; MCCONNELL *et al.* 1990; MCCONNELL and YAFFE 1993). One possible role for Bfr1p, given its role in secretion (see below), is to act as a link between the secretory apparatus and one of the three cytoskeletal systems. Another possible role for Bfr1p is in nuclear envelope dynamics. The nuclear membrane in *S. cerevisiae* does not break down during mitosis and therefore must double in size as the nucleus divides. Bfr1p may be involved in this process, as well as in ER and/or Golgi membrane function, thus explaining its effects on nuclear segregation and ER-to-Golgi transport.

Both *bfr1-Δ1::URA3 MATa* or *MATα* haploid and *bfr1-Δ1::URA3/bfr1-Δ1::URA3 MATa/MATα* diploid cells are considerably larger than the corresponding wild-type cells. Most of these mutant cells have a fairly normal morphology, except that the cells are more elongated than usual. Hence *bfr1-Δ1::URA3* mutants are not defective in polarized cell growth, characteristic of mutants in the actin cytoskeleton, which gives rise to

both small cells and large, round cells (DRUBIN 1990). This conclusion is supported by the observation that *bfr1-Δ1::URA3* cells have a normal distribution of actin (data not shown). A small fraction of *bfr1-Δ1::URA3* cells exhibit abnormal morphologies, and essentially all of these cells have abnormal nuclear morphologies. Hence it is possible that these cell morphology defects are a secondary consequence of the nuclear defects. Interestingly, the cell size and nuclear segregation phenotypes of *bfr1-Δ1::URA3* mutants are strain-dependent, since disruption of *BFR1* produced no visible phenotypes in the original strain that we tested. We have not determined whether these phenotypic differences are due to polymorphisms in only one or in several genes.

***BFR1* is implicated in secretion:** There are two pieces of evidence implicating *BFR1* in secretion. First, overexpression of *BFR1* partially suppresses the growth and secretion defects seen in the temperature-sensitive mutant *sec17* at a semipermissive temperature or in BFA-treated cells. Second, the *bfr1-Δ1::URA3* deletion allele enhances the partial growth and secretion defects of the temperature-sensitive *sec21* mutant at a semipermissive temperature.

One trivial explanation for the suppression of BFA-induced lethality by multicopy *BFR1* is that it simply enhances the permeability of the plasma membrane to BFA. We believe this explanation to be very unlikely, since it is difficult to explain how this effect would allow suppression of *sec17*. The conclusion that Bfr1p is implicated in secretion rather than membrane permeability is further strengthened by the fact that *BFR1* interacts genetically with *sec21*. A trivial explanation of the suppression of *sec17* by multicopy *BFR1* is that it elevates the permissive temperature of a strain nonspecifically. However, this possibility can be eliminated, since nine other temperature sensitive mutants were not suppressed by multicopy *BFR1*.

Our result that at the semipermissive temperature of 33°, *sec21* accumulates the late-Golgi p2 precursor of CPY, is the first evidence that *SEC21* is involved in intra-Golgi transport as well as ER-to-Golgi transport. This result is expected if *SEC21* indeed encodes a component of the non-clathrin coat that in mammalian cells is involved in both ER-to-Golgi and intra-Golgi transport (HOSOBUCHI *et al.* 1992; MELANÇON *et al.* 1991; ROTHMAN and ORCI 1992; STENBECK *et al.* 1992). We demonstrated that *sec21* is supersensitive to BFA, providing evidence that BFA disrupts the same step of transport as *sec21*, or that it inhibits a parallel pathway. This result links the target of BFA in yeast and the non-clathrin coat of Golgi membranes and vesicles (containing Sec21p/ γ -COP). The fact that deletion of the *BFR1* gene enhances the growth and secretion defects of *sec21*, whereas its overexpression partially suppresses the secretion defect imposed by BFA, provides another link between BFA action and Sec21p/ γ -COP in yeast. Although Sec17p is not a

component of the non-clathrin coat complex, it has been demonstrated that the mammalian homolog of Sec17p, α -SNAP, is a component of Golgi transport vesicles that must be assembled on the donor compartment membrane at the time of vesicle formation to produce fusion-competent vesicles (WATTENBERG *et al.* 1992). The genetic interactions between *BFR1* and both *SEC17* and *SEC21* may indicate that Bfr1p affects the membrane assembly of the Sec17p and Sec21p complexes at either the budding, targeting or fusion steps of vesicle transport.

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