Brefeldin A Causes a Defect in Secretion in Saccharomyces cerevisiae*

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Brefeldin A (BFA) blocks secretion in mammalian cells and causes the redistribution of Golgi resident membrane proteins to the endoplasmic reticulum (Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080). The target(s) of BFA and its mechanism of action remain unknown. The yeast Saccharomyces cerevisiae represents an ideal organism in which to identify the BFA targets, since many molecules essential for vesicular traffic have been already identified taking advantage of the powerful genetics of this system. Unfortunately, wild type S. cerevisiae strains are largely insensitive to BFA (Hayashi, T., Takatsuki, A., and Tamura, G. (1982) Agric. Biol. Chem. 46, 2241–2248). Here we demonstrate that an erg6 mutant (Ga-ber, R., Copple, D., Kennedy, B., Vidal, M., and Bard, M. (1989) Mol. Cell. Biol. 9, 3447–3456) defective in the biosynthesis of ergosterol is sensitive to BFA. Treatment of erg6 cells with BFA results in an arrest in growth and causes a block in secretion similar to that seen in mammalian cells treated with BFA. Our data suggest that the changes in the erg6 strain allows BFA entry and that this strain can be used to examine the molecular mechanism of BFA action.

A wide variety of compounds have been used to study the secretory pathway of eukaryotic cells. Recently, the fungal metabolite BFA has been shown to influence both the structure and the function of the organelles within the secretory pathway (1). In most mammalian cells, treatment with BFA results in a block in transport from the ER to the Golgi and a redistribution of Golgi resident proteins into the ER (1). Based on the observation that one of the earliest effects of BFA is a rapid redistribution of vesicle coat proteins (δ-coat protein, ADP-ribosylation factor, and γ-adaptin) and that coated vesicle budding is blocked in the presence of BFA, several groups have speculated that the target of BFA action may be the coat complex itself (4–8) or upstream effectors regulating assembly and disassembly of the coat complex. However, the molecules mediating the BFA effect remain to be identified and characterized.

Here we show that an erg6 strain of S. cerevisiae is sensitive to BFA. This provides a system that will allow the genetic and biochemical identification of the BFA target. Such information will be critical to our understanding of how BFA perturbs the normal secretory pathway in eukaryotic cells and thus provide insight into the molecular mechanism of vesicular traffic.

EXPERIMENTAL PROCEDURES

Materials—BFA was obtained from Sigma, Tran35S-label from ICN, and AMPLIFY from Amersham Corp. All other reagents were from Sigma.

Yeast Strains—Strains MY3117 (MATa ura3-52 his4-15 lys9 erg6) and MY3118 (MATa ura3-52 his4-15 lys9 erg6-Δ1) were kindly provided by Dr. Richard Gaber (Northwestern University, Chicago, IL). Strain MY3129 was constructed by transforming MY3118 with pMR1754, a plasmid containing invertase driven by the constitutive promoter TPI1.

Metabolic Labeling—Cells were labeled as described by Rose et al. (9) with the following modifications. Cells were grown to early exponential phase in minimal media. 4 × 10⁶ cells were harvested and washed in 2 ml of minimal medium lacking cysteine and methionine. Cells expressing invertase from the constitutive TPI1 promoter were treated with BFA and then radiolabeled. Cells expressing invertase from the inducible chromosomal gene were treated for 30 min in low glucose (0.1%) to derepress the synthesis of chromosomal invertase prior to BFA treatment and radiolabeling. BFA was added to a final concentration of 50 μg/ml. The cells were incubated in BFA for various amounts of time and then labeled for 20 min with 100 μCi of Tran35S-label in the presence of BFA. Labeling was terminated by addition of azide and cycloheximide to 10 mM and 100 μg/ml, respectively.

Pulse-chase experiments were performed as follows. Cells were grown to early exponential phase in minimal media and split into four aliquots. Two of the four aliquots were treated with BFA (50 μg/ml) for 10 min. All four samples were then labeled for 20 min with 100 μCi of Tran35S-label, and the incorporation of label was either terminated as before or the cells were incubated for 30 min in media containing excess (10-fold) of unlabeled methionine, cysteine, and ammonium sulfate (10).

The cells were separated into internal and external fractions following spheroplast preparation as described by Rothblatt et al. (10). Cell extracts were prepared as described by Rose et al. (9). Invertase and Kar2p were immunoprecipitated from radiolabeled samples, processed by SDS-polyacrylamide gel electrophoresis, and detected as previously described (11).

Electron Microscopy—Duplicate samples of wild type (MY3117) and erg6 (MY3118) strains grown to exponential phase in minimal media were treated with ethanol or with 50 μg/ml BFA in ethanol for 3 h. Cells were then pelleted and prepared for electron microscopy as described in Byers et al. (12).

RESULTS AND DISCUSSION

To examine the effect of BFA on wild type strain of S. cerevisiae (S288C), cells were streaked onto plates containing increasing amounts of BFA. As shown in Fig. 1A, BFA had no inhibitory action on the growth of S. cerevisiae, consistent with the report by Hayashi et al. (2) that S. cerevisiae is relatively insensitive to BFA. In fact, S. cerevisiae can grow on media containing greater than 20 times the BFA required to inhibit secretion in mammalian cells (1, 13). We postulated that the resistance might be due to the cell’s impermeability
to BFA. Therefore, we chose to examine the sensitivity of a strain defective in sterol biosynthesis based on the following two findings. First, econazole, a compound that blocks the synthesis of ergosterol, the major yeast sterol, causes yeast to have increased sensitivity to a wide variety of antibiotics (14). Second, the loss of ergosterol results in altered plasma membrane permeability to a large number of compounds. Consequently, we examined BFA sensitivity of a strain lacking ERG6 gene product (S-adenosylmethionine: Δ4-methyltransferase), an enzyme required for ergosterol synthesis (3). As predicted, the growth of erg6 cells was affected by 25 μg/ml BFA and was completely inhibited by concentrations greater than 50 μg/ml (Fig. 1A). Addition of 50 μg/ml BFA to a liquid culture resulted in a rapid block in growth and a slow decrease in viability (Fig. 1B).

BFA treatment of mammalian cells causes redistribution of Golgi proteins to the ER and results in Golgi-type glycosylation of resident ER proteins and secretory proteins blocked in transport out of the ER (1, 15). To test if BFA has a similar effect on yeast cells, the post-translational modifications of a well characterized yeast secretory protein, invertase, were analyzed. Yeast cells synthesize two forms of invertase: a constitutively expressed cytoplasmic form and an inducible secretory form (16). The normal invertase forms synthesized by induced cells include a 60-kDa cytoplasmic form, a minor 80-kDa core glycosylated form, and a 120–160 kDa heterogeneous population of mature invertase, which is the form secreted from the cells (Fig. 2A, lane 0 min). Pretreatment of cells with BFA for increasing length of time prior to metabolic labeling results in the rapid inhibition of mature invertase synthesis and the appearance of novel underglycosylated invertase form (Fig. 2A, lanes 10–90 min). This intermediate form of invertase is analogous to those produced in several yeast secretory mutants, including yot1 (17), arf1 (18), pmr1 (19), sec7 (16), and sec14 (16), which are defective in Golgi functions. The appearance of the intermediate form of invertase suggests that in yeast, as in mammalian cells, BFA blocks transport of proteins to the Golgi compartment in which terminal glycosylation occurs.

Exposure of erg6 cells to BFA for longer than 30 min resulted in a novel defect not yet documented for mammalian cells treated with BFA. Prolonged exposure resulted in the appearance of a form of invertase (62 kDa) having a mobility slightly slower than cytoplasmic invertase (Fig. 2A, lanes 30, 60, and 90 min). This form seems to be the precursor of secretory invertase containing an uncleaved signal sequence. This defect appears to be general since the processing of Kar2p, the yeast homologue of mammalian binding protein, was also affected (Fig. 2B). It is likely that the observed accumulation of unprocessed precursors is due to a block in translocation of proteins into the ER. In addition, BFA treatment resulted in increased synthesis of Kar2p. Kar2p has been shown to be induced under conditions of ER stress and our result is consistent with the recent finding that mammalian binding protein is also induced during BFA treatment (20).

To exclude the possibility that the BFA effect on the secretory pathway was due to a nonspecific “poisoning” of the cells, we examined the rates of protein synthesis in treated and untreated mutant cells. As shown in Fig. 2C, total protein synthesis was not affected by BFA treatment.

To characterize the secretory defect in greater detail, we examined whether the underglycosylated invertase synthet-
sized in the presence of BFA was retained within the *erg6* cell or was secreted. In contrast to the previous experiment, results presented in Fig. 3 are obtained with cells preincubated in low glucose to derepress the synthesis of secretory invertase from the chromosomal loci. After inducing invertase, the cells were treated with BFA, metabolically labeled, and processed to obtain internal and external fractions. It should be stressed that this induction-labeling regimen does not significantly influence overall protein synthesis. As shown in Fig. 3, mature invertase was efficiently secreted from wild type cells in the presence or absence of BFA (wild type panel). The secretory pathway of untreated *erg6* cells appears to be normal, since the kinetics of invertase secretion does not differ from that in the wild type cells. However, in *erg6* cells treated with BFA, approximately half of the underglycosylated form of invertase is not secreted even after a long chase. This phenotype is analogous to that seen with *ynl1* cells in which a proportion of newly synthesized invertase is retained within the cell (17). The block in secretion is dose-dependent, since increasing the concentration of BFA resulted in a higher percentage of invertase being retained within the cell (data not shown).

To determine if the underglycosylated intracellular invertase was retained within a compartment from which it could be subsequently secreted, the previous experiment was repeated with the modification that the BFA was removed at the beginning of the chase. Following removal of BFA, the majority of the retained invertase was rapidly secreted from the cell (Fig. 4), indicating that the intracellular invertase resides in a compartment from which it can be subsequently delivered to the cell surface. Thus in yeast, as in mammalian cells, the block in secretion caused by BFA is reversible. The form of invertase that was secreted was the immature partially glycosylated form lacking outer chains. These findings suggest that BFA-arrested invertase either accumulates in a compartment distal to the outer chain addition compartment or that it simply bypasses that compartment. Alternatively, BFA treatment might lead to a transient inactivation of an enzyme necessary for outer chain acquisition or to depression in sugar nucleotide pools. Further study will be required to elucidate this issue.

Treatment of mammalian cells with BFA affects the structure of the ER and Golgi compartments (1). To determine if BFA also alters the morphology of yeast subcellular compartments, BFA-treated *erg6* cells were examined by electron microscopy. *erg6* cells incubated with BFA accumulated long cisternal structures, probably ER, not abundant in untreated cells (Fig. 5, compare -BFA and +BFA panels). This morphology is similar to that shown for various secretory mutants blocked in ER to Golgi transport (21).

Although it is likely that the sensitivity of the *erg6* strain is the result of increased membrane permeability which allows entry of BFA, we cannot exclude other possibilities. For example, certain mammalian cell lines (e.g. PtK cells) are naturally resistant to BFA due to a non-diffusible factor associated with the Golgi (22). It is possible that BFA can enter wild type *Saccharomyces cerevisiae* cells and that their resistance to the drug is conferred by a similar Golgi-associated factor. If that were the case, then wild type *Saccharomyces cerevisiae* should remain resistant to BFA even if the drug can get into the cell. However, by simply increasing the concentration of BFA to very high levels (200 μg/ml) a partial defect in secretion similar to that seen with *erg6* cells treated with moderate levels of BFA (25 μg/ml) was observed in wild type cells (data not shown). This correlation between the effects seen in wild type cells treated with very high concentrations of BFA and those seen in *erg6* cells that have altered plasma membrane permeability strongly supports the hypothesis that entry of BFA is the limiting factor in analyzing BFA effects in yeast.

BFA has been used in many systems to study the membrane dynamics between the ER and the Golgi complex. Remarkably, these studies have led to the identification of a recycling pathway operational between the Golgi and the ER. Clearly, the identification and characterization of the BFA target(s) and the elucidation of its action will significantly increase our knowledge of how membrane traffic is controlled. Consequently, major effort has been directed to identify the BFA

### Fig. 3. BFA causes a block in secretion in an *erg6* strain.

The location of newly synthesized invertase was analyzed in wild type strain MY3117 and the *erg6* mutant strain MY3118. Following 30 min in low glucose to induce the synthesis of invertase, cells were treated with BFA for 10 min and then subjected to metabolic labeling for 20 min. One set of duplicate aliquots was then incubated with unlabeled methionine and cysteine for 30 min while still in the presence of BFA. Cells were harvested and separated into internal and external fractions. Internal fractions (*I*) represent material retained within the cell, and external fractions (*E*) represent material retained within the cell wall (the normal location of secretory invertase).

### Fig. 4. The effect of BFA is reversible. MY3118 (*erg6*-Δ1) cells were treated with BFA, metabolically labeled, and chased as in Fig. 3. At the end of the chase period, the BFA was removed and the cells were resuspended in fresh media for 0, 20, or 60 min. Cells were fractionated into internal (*I*) and external (*E*) fractions.

### Fig. 5. BFA treatment results in the accumulation of membranous structures in an *erg6* strain. Duplicate samples of wild type (MY3115) and *erg6* (MY3118) strains were treated with ethanol or with 50 μg/ml BFA in ethanol for 3 h. Cells were pelleted and prepared for electron microscopy as described (12). A, *erg6* cells treated with ethanol. B, *erg6* cells treated with BFA. BFA (50 μg/ml) had no effect on the wild type strain (data not shown).
target in eukaryotic cells. Recently, tritium-labeled BFA was used to label the major BFA-binding proteins in Chinese hamster ovary cells (23). Unfortunately, this direct biochemical approach failed to identify a likely BFA target. Rather, the major BFA-binding protein in Chinese hamster ovary cells is the enzyme glutathione S-transferase, which appears to be involved in BFA degradation (23). An alternative approach to identifying BFA targets would be the isolation of mutants resistant to BFA in a genetically tractable BFA-sensitive organism. The identification of a BFA-sensitive strain of S. cerevisiae, such as the erg6 mutant, will allow the use of the powerful combination of genetic and biochemical approaches, already used to elucidate functional components of the yeast secretory pathway, to study the underlaying mechanisms of BFA action in greater detail.

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