Brefeldin A blocks an early stage of protein transport in *Candida albicans*

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Brefeldin A (BFA) inhibited in a dose-dependent manner secretion of the cell-surface enzyme acid phosphatase (APase) into the periplasm of *Candida albicans* and caused intracellular accumulation of enzyme protein. Cells grown in the presence of BFA became more dense, implying that cell-surface growth was also blocked by BFA treatment. The APase that was accumulated intracellularly migrated faster on SDS-PAGE, suggesting less *N*-linked glycosylation compared with the mature, periplasmic APase produced in the absence of BFA. Pulse-chase experiments and gel-filtration of oligosaccharides released by Endo H treatment suggested that the core-glycosylated precursor form of APase accumulated in the presence of BFA. These results strongly suggested that endoplasmic reticulum (ER)-to-Golgi transport in *C. albicans* was inhibited by BFA. Aberrant membrane structures were observed in BFA-treated cells. Within 1 h of BFA removal these structures were replaced with rough ER membranes, suggesting that the accumulated membranes were derived from the ER.

Introduction

After synthesis on, and translocation across, the membranes of the rough endoplasmic reticulum (ER), secretory and membrane proteins are transported through the secretory pathway to their final destinations (Blobel & Dobberstein, 1975; Palade, 1975). The detailed mechanism of the vectorial transport of proteins along the distinct secretory organelles is obscure, but it is generally thought that interorganelle movement of proteins proceeds by budding of vesicles that carry proteins between cell compartments.

Movement of proteins from the ER to the Golgi apparatus is the first step in vesicular transport between separate membrane-bound organelles (Pfeffer & Rothman, 1987). It is also often the rate-limiting step in the secretion of proteins (Lodish *et al.*, 1983; Warren, 1987). In addition, initial protein sorting has to be carried out in order to selectively retain resident ER proteins (Munro & Pelham, 1987; Pelham, 1988; Tartakoff & Vassalli, 1978). Clarifying the underlying mechanism of this process is one of the central issues in the study of protein secretion.

To date, several methods have been developed to study ER-to-Golgi transport processes *in vivo* and *in vitro*. First,

biochemical and histochemical techniques which combined autoradiography and cell fractionation revealed the general pathway of protein secretion, starting from the rough ER (Palade, 1975). It was also demonstrated that ER-to-Golgi transport could be inhibited by an inhibitor of oxidative phosphorylation (Jamieson & Palade, 1968). Secondly, a genetic approach using temperature-sensitive secretory (*sec*) mutants of *Saccharomyces cerevisiae* demonstrated the involvement of more than 10 gene products in ER-to-Golgi transport process (Novick & Schekman, 1979; Schekman, 1985). Thirdly, *in vitro* systems that reconstituted ER-to-Golgi protein transport were developed by employing semi-permeabilized mammalian and yeast cells (Baker *et al.*, 1988; Beckers *et al.*, 1987; Haselbeck & Schekman, 1986; Ruohola *et al.*, 1988; Simons & Virta, 1987). These experiments have shown the existence of some factor(s) involved in ER-to-Golgi protein transfer as well as in other transport processes.

The use of specific inhibitors is another powerful means for analysing the mechanism of protein export (Griffiths *et al.*, 1983; Tartakoff & Vassalli, 1977, 1978). It can help to dissect the individual steps of the secretory pathway and therefore may be expected to reveal hitherto unknown aspects of intracellular protein transport. For example, morphological and biochemical analyses of the effect of the ionophore monensin have demonstrated that the acidic pH on the *trans*-side of the Golgi apparatus is necessary for protein transport from *medial*- to *trans*-Golgi (Griffiths *et al.*, 1983).

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Abbreviations: APase, acid phosphatase; BFA, Brefeldin A; Endo H, endoglycosidase H; ER, endoplasmic reticulum.

Brefeldin A (BFA) is a macrolide antibiotic produced by some fungi (see references in Hayashi *et al.*, 1974). We have previously shown that BFA specifically inhibits intracellular transport of vesicular stomatitis virus (VSV) G protein in mammalian cells (Takatsuki & Tamura, 1985). From biochemical analyses it was concluded that BFA interfered with transport before, or at, the *cis*-most cisterna of the Golgi apparatus. The aim of this study was to determine if BFA exerts the same effect in yeasts as it does in mammalian cells. For this purpose the effect of BFA on the secretion of a periplasmic enzyme, acid phosphatase (APase) in *Candida albicans* was examined.

Methods

Organism and materials. *Candida albicans* IAM 4888 was used throughout this study. YEPD medium contained 1% (w/v) Yeast Extract (Difco), 2% (w/v) peptone (Difco) and 2% (w/v) glucose. For induction of APase, KH_2PO_4 was replaced by equimolar (11 mM) KCl in Burkholder medium (Burkholder, 1943). Glucose was replaced by galactose for efficient incorporation of [^3H]mannose.

BFA was purified from cultures of a fungus isolated from soil samples as reported previously (Tamura *et al.*, 1968). Zymolyase 100T and endoglycosidase H (Endo H) were obtained from Seikagaku Kogyo Co. IgG sorb was from The Enzyme Center, Sephadex G-100 was from Pharmacia, and PMSF, leupeptin and pepstatin A were from Sigma. [^3S]Methionine [1113 Ci mmol $^{-1}$ (41 TBq mmol $^{-1}$)] was from Muromachi Kagaku Co. [^3H]Mannose [40.6 Ci mmol $^{-1}$ (1.5 TBq mmol $^{-1}$)] was from Daiichi Kagaku Co. All other reagents used were of analytical grade.

APase induction and the measurement of enzyme activity. APase was induced as described by Mizunaga (1979). Briefly, *C. albicans* IAM 4888 grown overnight at 30 °C to exponential phase in YEPD medium ($\text{OD}_{550} = 2$) was harvested by centrifugation (700 g, 5 min), washed twice with distilled water and suspended in Burkholder medium without inorganic phosphate ($\text{OD}_{550} = 1$). BFA (methanolic solution) was added to give the indicated concentrations at the start of induction and was present throughout the following procedures. After incubation with shaking (150 r.p.m.) at 30 °C for the times indicated cells were harvested, washed twice and suspended in protoplast buffer (10 mM-Tris/HCl, pH 7.5, 1.4 M-sorbitol, 5 mM-MgCl $_2$ and 10 mM-Na $_2\text{N}_3$). Zymolyase 100T (100 $\mu\text{g ml}^{-1}$) and 2-mercaptoethanol (20 mM) were added and the suspension was incubated for 45–60 min. Protoplasts formed during this period were separated from the periplasm by centrifugation (1500 g, 5 min), washed twice with the protoplast buffer and disrupted by sonication (Branson Cell Disruptor 200) for 20 s on ice. The insoluble material was removed by centrifugation at 8000 g for 5 min. The supernatant (protoplast lysate) and the periplasm fractions were assayed for APase activity as described by Mizunaga (1979). Values are expressed as units of APase activity per OD_{550} of cells.

Percoll density gradient. Cells treated with the designated concentrations of BFA for 3 h in YEPD medium were harvested, suspended in 2 ml of ice-cold distilled water and loaded onto the top of 10 ml 90% (v/v) Percoll in a Hitachi 13 PA tube. After centrifugation in a Hitachi RPS 40T rotor at 35000 r.p.m. for 30 min, fractions were taken from the bottom of the tube by using a peristaltic pump. Samples from each fraction were appropriately diluted and spread on YEPD agar plates. The viable cell number of each fraction was determined and expressed

as a percentage of the total number of cells detected either for the control or for each of the BFA concentrations.

Anti-APase antiserum. APase was purified from a *C. albicans* cell culture as described by Odds & Hierholzer (1973) and used as immunogen to raise anti-APase antiserum in rabbits. The serum obtained had a titre of >1:2000 in an ELISA reaction and could immunoprecipitate APase activity.

Radiolabelling and immunoprecipitation. After induction of APase for 2 h in phosphate-depleted medium as described above, cultures were incubated with or without BFA (10 $\mu\text{g ml}^{-1}$) for a further 15 min. [^3S]Methionine [5 $\mu\text{Ci ml}^{-1}$ (185 kBq ml $^{-1}$)] was then added and the cells were incubated for a further 3 h. Labelled cells were harvested, washed, treated with Zymolyase and separated into periplasm and protoplast fractions as described. Protoplasts were lysed and centrifuged at 100000 g for 1 h to remove insoluble materials. Periplasm and protoplast fractions were incubated with anti-APase polyclonal antiserum for 14 h at 0 °C. IgG sorb (10%, w/v, suspension; half the volume of antiserum) was added to the immunoreaction mixture, incubated on ice for 30 min with gentle shaking and centrifuged at 12000 r.p.m. for 1 min. The supernatant was removed and the precipitate was washed five times with immunoprecipitation buffer (100 mM-potassium phosphate buffer, pH 7.4, 150 mM-NaCl, 1%, w/v, sodium deoxycholate, 0.05% SDS, 0.5% Triton X-100 and 0.5 mM-PMSF). The final precipitates were boiled for 3 min in SDS sample buffer and analysed by SDS-PAGE (10%, w/v, acrylamide). The dried gels were enhanced and bands were visualized by fluorography at –80 °C.

In pulse-chase experiments, cells incubated in phosphate-depleted medium for 2 h were pretreated with or without BFA for 15 min, labelled with 500 μCi (18.5 MBq) [^3S]methionine ml $^{-1}$ for 10 min and chased by adding unlabelled methionine to 30 mM. At the times indicated samples of cells were removed and frozen immediately in a solid CO $_2$ /ethanol bath. After freezing and thawing at least five times, cells were treated with Zymolyase, lysed and immunoprecipitated as described.

The recovery of APase secretion was monitored using cells pretreated with BFA, labelled with [^3S]methionine for 60 min in the presence of BFA as described above, and then washed and chased in BFA-free medium containing 30 mM unlabelled methionine. Samples were removed at the recovery times indicated and processed as described above.

Endo H treatment and gel filtration. Immunoprecipitated APase was treated with Endo H as follows. The immunoprecipitates were boiled for 3 min in 20 μl 1% (w/v) SDS. Samples were cooled, adjusted to 20 mU Endo H ml $^{-1}$, 1 mM-PMSF, 20 $\mu\text{g ml}^{-1}$ each of leupeptin and pepstatin A, 0.02% (w/v) Na $_2\text{N}_3$ and 0.1% SDS in 0.5 M-sodium citrate buffer, pH 5.5, and incubated for 16 h at 37 °C.

For efficient incorporation of [^3H]mannose into saccharide chains, the carbon source of Burkholder medium was changed from 2% glucose to 0.5% galactose. After APase had been induced in this medium for 2 h, cells were labelled with 1 μCi (37 kBq) [^3H]mannose ml $^{-1}$ for 3 h in the presence or absence of 10 $\mu\text{g ml}^{-1}$ BFA. Cells were lysed by Zymolyase and sonication treatment, immunoprecipitated and treated with Endo H as above. The Endo-H-digested samples were boiled for 5 min, centrifuged (12000 g, 10 min) and the final supernatant was applied to a Sephadex G-100 column (1.1 $\times$ 45 cm), pre-equilibrated and eluted with 150 mM-NaCl. Fractions of 1 ml were collected, dried on Whatman GF/C filters and the radioactivity was measured. Molecular mass calibration was done with blue dextran, bovine serum albumin (69 kDa), ovalbumin (45 kDa), lysozyme (14.3 kDa) and [^3H]mannose as standards.

Electron microscopy. Preparation of thin sections of yeast cells by freeze-substitution done as described by Tanaka & Kanbe (1986) with

minor modifications. Cells grown in the presence or absence of $10 \mu\text{g BFA ml}^{-1}$ in Burkholder medium for 10 h were collected on copper meshes and plunged into liquid propane cooled with liquid N_2 . Frozen cells were transferred to 2% (w/v) OsO_4 in anhydrous acetone, and kept at -80°C for 48 h with solid CO_2 /acetone. They were transferred to -35°C for 2 h, 4°C for 2 h and then to room temperature for 2 h. After washing three times with absolute acetone, samples were infiltrated with increasing concentrations of Spurr's resin in absolute acetone and finally with 100% Spurr's resin. They were then polymerized in capsules at 50°C for 5 h and 70°C for 30 h. Thin sections cut with a Sorvall MT-2 ultramicrotome were collected, stained with uranyl acetate and lead citrate and observed in a JEOL 200 CX electron microscope at 100 kV.

Results

BFA inhibits protein secretion in *C. albicans*

Most of the studies of the mechanism of protein secretion in yeasts have used *Saccharomyces cerevisiae* (Schekman, 1985). This organism is relatively insensitive to BFA, making it an unsuitable organism for analysing the action of BFA. In contrast, previous work showed that *Candida albicans* IAM 4888 was the most sensitive of the yeasts tested to BFA (Hayashi *et al.*, 1982). Thus we selected this strain of *C. albicans* to study the action of BFA on protein secretion in yeast.

The effect of BFA on the secretion of the *C. albicans* cell-surface glycoprotein APase was examined. As shown in Fig. 1(a), BFA inhibited secretion of APase in a dose-dependent manner. At the highest BFA concentration tested ($10 \mu\text{g ml}^{-1}$), APase secretion was only one-tenth that of untreated control cells. In contrast, a several-fold increase in intracellular APase activity was observed in BFA-treated cells compared with that of the controls, indicating intracellular accumulation of the enzyme (Fig. 1b). BFA had little effect on the incorporation of labelled leucine into TCA-insoluble fractions (Hayashi *et al.*, 1982, and our unpublished data), which suggests that decrease in periplasmic APase activity was due to the inhibition of APase secretion rather than the inhibition of protein synthesis. Several possibilities could be considered to account for the accumulation of less APase at 5 and $10 \mu\text{g BFA ml}^{-1}$ than at $2.5 \mu\text{g ml}^{-1}$. For example, BFA might cause selective inhibition of secretory protein synthesis, or accumulation of inactive forms of APase and degradation of APase accumulated within the cells.

Transport of plasma-membrane proteins to the cell surface is known to depend on the secretory pathway as is the discharge of proteins into the periplasm or culture medium (Brada & Schekman, 1988). If intracellular protein transport is blocked by BFA, plasma-membrane proteins are unable to reach the cell surface and therefore accumulate within the cells, resulting in severe decrease

Fig. 1. Inhibition of APase secretion by BFA. *C. albicans* IAM 4888 was grown in phosphate-depleted medium in the presence of the indicated concentrations of BFA. After 5 h cells were harvested and converted to spheroplasts by treatment with Zymolyase; APase activities in both periplasm (a) and protoplast lysate (b) fractions were then determined. Values are expressed as percentages of the control; 100% activity = $9.6 \times 10^{-2} \text{ U (OD}_{550})^{-1}$. Points represent single determinations of APase activity. The experiment was repeated three times with essentially the same results.

or cessation of net cell-surface growth. Since synthesis of proteins, DNA and RNA is hardly affected by BFA treatment (Hayashi *et al.*, 1982), continuous synthesis of macromolecules may be expected to increase the density of BFA-treated cells. To test this possibility, we looked for changes in cell density after BFA treatment. Cells incubated in the presence or absence of BFA for 3 h were loaded onto 90% Percoll, centrifuged and fractionated; the viable cell numbers in each fraction were then determined as described in Methods. As shown in Fig. 2, viable cell counts in the bottom fraction (fraction no. 1) increased after BFA treatment, indicating that cells grown in the presence of BFA became more dense than untreated control cells. The total number of viable cells was essentially the same at each BFA concentration. The proportion of dense cells was increased by raising the concentration of BFA, suggesting that inhibition of transport, incomplete at lower BFA concentrations, gradually became greater with increasing BFA concentration. This dose-dependency is in good accord with the effect shown above on secretion of APase. Taken together, these results suggest that BFA inhibits protein secretion in *C. albicans* and, consequently, causes intracellular accumulation of proteins normally destined for the cell surface, resulting in an increase in cell density.

Fig. 2. Percoll density gradient centrifugation of BFA-treated and untreated cells. Cells incubated in the presence of the indicated concentrations of BFA for 3 h in YEPD medium were collected and loaded onto 90% Percoll. After centrifugation, fractions were collected from the bottom and spread on YEPD agar plates. The number of colonies was counted after 2 d. BFA concentrations ($\mu\text{g ml}^{-1}$): $\circ$, 0; $\triangle$, 2; $\square$, 4; $\blacktriangle$, 6; $\bullet$, 10. Points represent single determinations of viable cell counts. The experiment was repeated twice with essentially the same results.

The effect of BFA is rapid and reversible

In *sec* mutants of *S. cerevisiae*, secretion of cell-surface glycoproteins stops immediately upon incubation at the restrictive temperature (Novick *et al.*, 1980, 1981). The transport block is reversible, accumulated glycoproteins being secreted to the periplasm on return to the permissive temperature. A similar rapid and reversible effect was observed with BFA. Upon addition of $10 \mu\text{g ml}^{-1}$ BFA, secretion of APase into the periplasm stopped immediately and simultaneously APase began to accumulate within the protoplasts (Fig. 3*a, b*). After BFA treatment for 2 h, cells were collected, washed and resuspended in fresh, BFA-free medium. Secretion of APase quickly resumed after the removal of BFA and the amount of APase accumulated within the protoplasts began to fall with little lag period (Fig. 3*a, b*). Thus the inhibition of secretion caused by BFA was very rapid and almost completely reversible.

An early stage of intracellular transport is blocked by BFA

N-Linked oligosaccharide moieties of glycoproteins are assembled sequentially within distinct compartments of the secretory pathway (Ballou, 1976; Byrd *et al.*, 1982; Esmon *et al.*, 1981). Core-glycosylation and trimming of three glucose residues and one mannose residue take place in the ER while bulky outer-chain carbohydrates

Fig. 3. Rapid and reversible effect of BFA. After induction of APase for 3 h cells were treated for 2 h with BFA ($10 \mu\text{g ml}^{-1}$) ($\blacktriangle$) or were left untreated ($\circ$). Treated cells were separated into two equal portions: one half was further incubated in the presence of BFA ($\blacktriangle$), the other was quickly washed twice with distilled water and incubated in the absence of BFA ($\triangle$). After 0, 5, 10, 20, and 60 min cells were collected, converted to protoplasts and APase activities in both periplasm (*a*) and protoplast lysate (*b*) fractions were determined. Points represent single determinations of APase activity. The experiment was repeated twice with essentially the same results.

are added to core oligosaccharides in the Golgi apparatus. Thus the extent of modification of N-linked carbohydrates can be used to determine the location of the protein of interest. To assess the site of inhibition of intracellular transport and eventual accumulation of APase caused by BFA treatment, we compared the degree of APase glycosylation in the presence or absence of BFA by immunoprecipitation followed by SDS-PAGE.

In the periplasm fraction of the control, an intense smeared band of secreted, mature-form APase was present, migrating with an apparent molecular mass of 120–150 kDa (Fig. 4, lane 4; Odds & Hierholzer, 1973). In contrast, in the BFA-treated cells APase could not be detected in the periplasm but only in the protoplast fraction, again indicating inhibition of secretion and intracellular accumulation of APase (Fig. 4, lanes 9 and 7, respectively). In the latter case, the APase migrated with an apparent molecular mass of 96 kDa, which is considerably smaller than that of mature APase. This difference in molecular mass is presumably due to the extent of N-linked glycosylation, since Endo H treatment converted APase from both BFA-treated and untreated cells to proteins of 76 kDa and 51 kDa (Fig. 4, lanes 5 and 8, respectively). The 51 kDa band and the lower molecular mass band in lane 7 may be degradation products. Since heterogeneous electrophoretic mobility of mature APase is thought to be attributable to the addition of outer-chain carbohydrates in the Golgi

Fig. 4. Immunoprecipitation of APase in BFA-treated and untreated cells. Cells induced to synthesize APase for 2 h were labelled with $5 \mu\text{Ci}$ (185 kBq) [^{35}S]methionine ml^{-1} in the presence or absence of $10 \mu\text{g}$ BFA ml^{-1} for 3 h. They were converted to protoplasts and APase in the periplasm and the protoplast lysate fractions was immunoprecipitated, treated with Endo H and analysed by SDS-PAGE.

apparatus, it would be expected that the 96 kDa species of APase, which migrated relatively homogeneously and accumulated in the presence of BFA, does not contain outer-chain carbohydrates but is modified only by core oligosaccharides. An indirect examination of this possibility was made in a pulse-chase experiment (Fig. 5). The 96 kDa species was the most abundant form of APase after a 10 min labelling period in both control and BFA-treated cells (Fig. 5, lanes 1 and 7). In the control the amount of this species of APase gradually decreased as it was converted during the chase to mature APase with lower and heterogeneous electrophoretic mobility, suggesting that the 96 kDa species is an intermediate in the formation of the highly glycosylated mature APase (Fig. 5, lanes 1–6). However, when the pulse-chase experiment was done in the presence of BFA, conversion of the 96 kDa species to the mature form was not observed, the amount of the 96 kDa species remaining relatively constant throughout the chase period (Fig. 5, lanes 7–12). When BFA-treated cells were washed and re-incubated

in BFA-free medium, this 96 kDa species appeared to be converted to highly glycosylated mature APase as observed in the control (Fig. 5, lanes 13–15). These results suggest that the 96 kDa species that accumulated in the presence of BFA is the APase precursor which is detected immediately after pulse-labelling. The rapid radiolabelling of the 96 kDa species with homogeneous electrophoretic mobility and its subsequent conversion to diffusely migrating mature APase, which seems to contain outer-chain carbohydrates, strongly suggest that the 96 kDa species is the core-glycosylated precursor of APase.

To confirm that the 96 kDa species is indeed the core-glycosylated form of APase, an estimation was made of the molecular masses of the *N*-linked carbohydrates liberated by Endo H treatment. APase was labelled with [^3H]mannose in the presence or absence of BFA, immunoprecipitated and digested with Endo H. Released carbohydrate moieties were then passed through a Sephadex G-100 gel filtration column and the radio-

Fig. 5. Pulse-chase experiments. Cells induced to synthesize APase were pulse-labelled in the presence or absence of $10 \mu\text{g BFA ml}^{-1}$ with $500 \mu\text{Ci (18.5 MBq) } [^3\text{S}]\text{methionine ml}^{-1}$ for 10 min and chased by adding 30 mM-methionine. At the times indicated samples were collected and treated as described in Methods. Lysed cell extracts were immunoprecipitated and analysed by SDS-PAGE. For experiments on the recovery of APase secretion (lanes 13–15), cells were labelled in the presence of BFA for 60 min, and quickly washed twice and chased in the absence of BFA.

activity in each fraction was measured (Fig. 6). In the control, the major peak of *N*-linked saccharides was eluted near the void volume of the column, fraction no. 18, and its molecular mass was determined to be 25–40 kDa. In contrast, in BFA-treated cells the major peak appeared around fraction no. 44, which corresponds to a molecular mass of 2.5–4 kDa (Fig. 6), close to the molecular mass of the core oligosaccharide (about 2 kDa). Although structural analysis of the oligosaccharide in this peak fraction was not done, the results suggest that the 96 kDa species of APase that accumulates in BFA-treated cells has acquired core oligosaccharides but not outer-chains. Note that as proteins were used as molecular mass standards, the molecular mass values that we quote for carbohydrates are approximate.

Since core oligosaccharides are added to exported proteins in the ER, the fact that the core-glycosylated 96 kDa species of APase accumulated intracellularly as a result of BFA treatment again strongly suggests that APase was retained in the ER or in the compartments before the Golgi apparatus, where outer-chain carbohydrates are assembled. Thus we propose that in *C. albicans*

Fig. 6. Gel-filtration of *N*-linked saccharides of APase. APase induced in Burkholder medium containing 0.5% galactose was labelled with $1 \mu\text{Ci (37 kBq) } [^3\text{H}]\text{mannose ml}^{-1}$ for 3 h in the absence (○) or presence (●) of $10 \mu\text{g BFA ml}^{-1}$. Lysed cell extracts prepared by Zymolyase treatment were immunoprecipitated and digested with Endo H. Released *N*-linked saccharide chains were applied to a Sephadex G-100 column, fractionated, and the radioactivity in each fraction was determined. Similar results were obtained in two experiments with samples from independent cultures.

Fig. 7. Electron micrographs of BFA-treated and untreated cells. (a) Control *C. albicans* cells. (b) Marked proliferation of internal membrane structure in cells treated with BFA ($10 \mu\text{g ml}^{-1}$) for 10 h. (c, d) Cells treated with BFA for 10 h. Aberrant membranes are continuous with the outer nuclear membrane (in c, N marks the nucleus). Stacks of membrane structures were formed on the luminal side of the proliferated membrane (in d, arrowheads). (e, f) Concentric membrane structure observed in BFA-treated cells. Membranes were closely packed together and the central region of the structure was filled with ribosome-like particles. Bars: $1 \mu\text{m}$ in (a), (b) and (e); $0.5 \mu\text{m}$ in (c), (d) and (f).

Fig. 8. Recovery of morphology of intracellular membrane structure after removal of BFA. Cells treated with BFA for 10 h were washed and re-incubated in BFA-free medium for 1 h. Ribosome-attached membranes are observed just beneath the plasma membrane, which is typical of the location and morphology of yeast ER membranes. Bars, 0.5 μ m.

BFA blocks intracellular protein transport between the ER and the Golgi apparatus, as in animal cells.

BFA treatment induces aberrant intracellular membrane structures

Observation by light microscopy demonstrated that the morphology of BFA-treated cells changed from the typical ovoid form to a spherical shape, which was always accompanied by changes in refractivity. BFA also caused the cell volume to increase to two to three times that of the control cells (Hayashi *et al.*, 1982). In order to examine the morphological changes at the ultrastructural level, cells fixed and processed by freeze-substitution (Tanaka & Kanbe, 1986) were observed by electron microscopy.

As shown in Fig. 7(a), in thin sections of control cells internal membranes other than nuclear, mitochondrial and vacuolar membranes were rarely seen. On the other hand, cells treated with BFA for 10 h showed dramatic accumulation of membrane structures of unknown origin (Fig. 7b–f). These structures were found throughout the cytoplasm and most of them were smooth, ribosome-free membranes. They were, at least in some places, continuous with the outer nuclear membrane (Fig. 7c), which suggests that they are derived from the ER. In some cells the luminal side of these membrane structures expanded and enclosed stacks of membranes (Fig. 7d). Occasionally, an unusual membrane structure was observed in which more than ten membrane units were assembled into a concentric circle (Fig. 7e, f). The membranes were very closely packed with little space between them and in the centre of these concentric membranes were a number of small particles, presum-

ably ribosomes, suggesting that these membranes are surrounding cytoplasm.

The effect of BFA is reversible and secretion resumes readily after removal of BFA (Fig. 3). We next examined if the BFA-induced morphological changes were also reversible. Cells treated with BFA for 10 h were washed and re-incubated in BFA-free medium for 1 h. As shown in Fig. 8(a, b), the irregularly shaped membranes that accumulated in the presence of BFA largely disappeared and ribosome-bound membranes were observed just beneath the cell membrane, which is the typical location and morphology of yeast ER membranes. Cells incubated for longer periods (10 h) showed the normal appearance, that is, the ER membranes were rarely observed. Thus the morphological changes caused by BFA could be reversed, consistent with the reversible effect of BFA on APase secretion.

Discussion

In *S. cerevisiae*, modifications of the *N*-linked carbohydrate moieties of mannoproteins are carried out sequentially in transit through the secretory pathway (Byrd *et al.*, 1982; Esmon *et al.*, 1981, 1984). Core oligosaccharide units consisting of GlcNAc₂Man₉Glc₃ are added to the nascent polypeptide chains in the ER where they are processed to GlcNAc₂Man₈. After delivery to the Golgi apparatus *N*-linked sugars are elongated by addition of bulky outer-chain carbohydrates (Ballou, 1976). Due to their heterogeneity, glycoproteins transported through the Golgi apparatus exhibit a wide range of electrophoretic mobilities. Analysis of APase accumulated in BFA-treated *C. albicans* cells suggested that it was the core-

glycosylated precursor form, which meant that it had not reached the Golgi apparatus. Thus it is probable that in *C. albicans* BFA inhibits protein transport between the ER and the Golgi apparatus, as in animal cells.

BFA-treated cells accumulated abnormal membrane structures (Fig. 7*b–f*). The origin and nature of these membranes are unknown, but they have some resemblance to the accumulated network of ER membranes seen in some ER-exaggerating *sec* mutants (Novick & Schekman, 1979; Novick *et al.*, 1980, 1981) and they are also reminiscent of structures observed in the cold-sensitive *ypt1-1* mutant of *S. cerevisiae* (Schmitt *et al.*, 1988). Considering that these mutants have defects in transport from the ER to the Golgi apparatus, together with the fact that BFA inhibits a similar step in intracellular protein transport in *C. albicans* and in animal cells, it can be speculated that the accumulated membrane structures of BFA-treated cells are an abnormally proliferated version of the ER. The observations that at least some portions of accumulated membranes are continuous with the outer-nuclear membrane and that significant amounts of ribosome-attached membranes, that are rarely seen in control cells, appeared during recovery from BFA treatment, support this. Furthermore, our unpublished observations that the membranes accumulated in BFA-treated cells are little or weakly stained by the periodic acid/thiocarbohydrazide/silver protein method, which specifically stains 'Berkely bodies' in *sec7* mutants (Novick *et al.*, 1980, 1981), indicate that these membranes are not derived from the Golgi apparatus.

A small percentage of BFA-treated cells contained concentric membrane structures made up of more than 10 layers of packed membranes (Fig. 7*e, f*). It is unlikely that these are artifacts generated in the process of specimen preparation, since the freeze-substitution method used is thought to be the most suitable procedure for retaining the features of intact cells. Similar remarkable proliferation of stacked concentric membrane structures was observed in animal and yeast cells by Orci *et al.* (1984) and by Wright *et al.* (1988). In both these cases overproduction of HMG-CoA reductase, an integral membrane protein of the ER, resulted in marked accumulation of membrane structures called 'crystalloid ER' and 'karmellae', respectively. However, it is unlikely that the appearance of concentric membranes in BFA-treated cells is the direct consequence of overproduction of HMG-CoA reductase, since no increase in HMG-CoA reductase activity was observed in BFA-treated cells (unpublished data).

Recent studies from two laboratories (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989) focused on the Golgi apparatus as the target organelle of BFA action in mammalian cells. One possible explanation is that BFA

causes the *cis*- and *medial*-Golgi cisternae to vesiculate, disappear and fuse inappropriately with the ER, so that eventually protein transport will cease due to the absence of the Golgi apparatus. If this is the case with *C. albicans*, then the Golgi membranes that fused with the ER membranes did not retain Golgi-like characteristics. The rare existence in control *C. albicans* cells of the typical Golgi structures seen in animal and plant cells prevented us from directly comparing morphological changes of the Golgi apparatus caused by BFA in yeast.

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