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# Rapid Redistribution of Golgi Proteins into the ER in Cells Treated with Brefeldin A: Evidence for Membrane Cycling from Golgi to ER

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## Summary

In cells treated with brefeldin A (BFA), movement of newly synthesized membrane proteins from the endoplasmic reticulum (ER) to the Golgi apparatus was blocked. Surprisingly, the glycoproteins retained in the ER were rapidly processed by *cis*/medial Golgi enzymes but not by *trans* Golgi enzymes. An explanation for these observations was provided from morphological studies at both the light and electron microscopic levels using markers for the *cis*/medial and *trans* Golgi. They revealed a rapid and dramatic redistribution to the ER of components of the *cis*/medial but not the *trans* Golgi in response to treatment with BFA. Upon removal of BFA, the morphology of the Golgi apparatus was rapidly reestablished and proteins normally transported out of the ER were efficiently and rapidly sorted to their final destinations. These results suggest that BFA disrupts a dynamic membrane-recycling pathway between the ER and *cis*/medial Golgi, effectively blocking membrane transport out of but not back to the ER.

## Introduction

One of the most characteristic features of the vacuolar system through which secretory and plasma membrane proteins travel is that it is composed of an ordered array of membrane-bound compartments. Newly synthesized proteins enter this system in the rough endoplasmic reticulum (ER) and then travel in a vectorial fashion through the *cis*, medial, and *trans* Golgi cisternae to a complex organelle system referred to as the *trans* Golgi network. From here they are routed to lysosomes, secretory granules, or the plasma membrane via vesicles that either mature into or fuse with those end-stage organelles. Each organelle in this pathway has distinct characteristics, components, and functions. Specific processing enzymes that catalyze reactions within these organelles modify proteins to create signatures that they have reached a particular destination along the pathway.

The massive movement of proteins along this pathway raises the question of how the individual organelles along the pathway maintain their distinctive properties in the face of a continuous flow of membrane into and out of each compartment. Not only must this dynamic state allow the retention of unique resident proteins, but there must also be a mechanism to preserve the total mem-

brane content and surface area of each organelle. These problems are particularly significant for the ER, for which bulk flow measurements by Rothman and colleagues suggest that half the lipids are removed every 10 min without a comparable rate of *de novo* synthesis (Wieland et al., 1987). Two possibilities have been suggested for how lipids are returned to the ER: from a post-ER compartment (most likely the *cis* Golgi) by vesicular recycling (Rothman, 1981; Farquhar, 1985), or by the cytosolic transfer of non-vesicular lipid from Golgi to ER (Wieland et al., 1987). Vesicular recycling would have important implications for the mechanism(s) for retention of ER proteins (Warren, 1987). Resident ER proteins might not be merely anchored to the ER and retained in the face of an enormous flow of membrane into and out of the ER, as a nonvesicular lipid recycling model would predict. Instead, selective retrieval of retained proteins from the *cis* Golgi back to the ER might occur, as suggested by one recent study by Pelham (1988).

To learn more about the mechanisms regulating membrane flow to and from the ER, we have used brefeldin A (BFA), a fungal antibacterial reagent reported to inhibit the transport of proteins out of the ER (Misumi et al., 1986). In this study we demonstrate that, in addition to preventing proteins from leaving the ER, treatment of cells with BFA has a profound effect on the structure of the *cis*/medial Golgi. Within minutes of BFA treatment, *cis*/medial Golgi components redistribute to and become part of the ER. Subsequent to this redistribution, processing of the carbohydrate chains of ER-retained glycoproteins by *cis*/medial Golgi enzymes is observed. A *trans* Golgi marker, in contrast, does not show redistribution to the ER during BFA treatment. We also observe no carbohydrate processing of ER-retained proteins that could be attributed to the *trans* Golgi. The effect of BFA is rapidly reversed upon removal of the drug. Minutes after the removal of BFA, Golgi proteins are rapidly transported from the ER into a newly reforming Golgi apparatus while ER-resident proteins with which they had been mixed remain in the ER. In addition, exportable proteins recover their ability to be transported out of the ER, through the Golgi apparatus, and onto more distal target organelles. These results can most simply be explained by a model in which BFA interferes with a dynamic membrane-recycling pathway between the ER and the *cis*/medial Golgi.

## Results

We have used the T cell antigen receptor (TCR) to study the effects of BFA on intracellular protein transport. This receptor is a multimeric protein complex composed of at least seven transmembrane chains. The  $\alpha$  and  $\beta$  chains form a disulfide-linked heterodimer that functions in antigen recognition. They are noncovalently associated with five other chains:  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and the homodimer  $\zeta_2$  (Samelson et al., 1985). Four of the TCR subunits ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ) contain N-linked carbohydrate chains. Newly synthe-

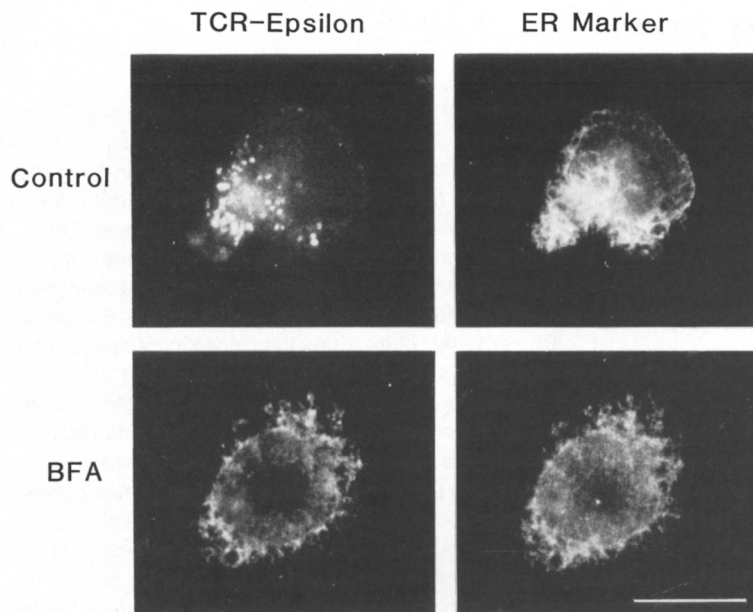


Figure 1. Immunofluorescence Localization of TCR  $\epsilon$  Chains and ER-Resident Proteins in BFA-Treated and Untreated T Cells

2B4 cells were incubated for 3 hr in the presence or absence of BFA (10  $\mu\text{g/ml}$ ) at 37°C. Cells were fixed in formaldehyde, permeabilized with saponin, and incubated with anti- $\epsilon$  antibodies and antibodies against ER-resident proteins. After washing to remove unbound IgG, cells were incubated with rhodamine-labeled goat anti-hamster IgG to label the specifically bound anti- $\epsilon$  IgG and with fluorescein-labeled goat anti-rabbit IgG to label specifically bound ER-directed antibodies. After a final wash the cells were prepared for microscopy. (Upper left and right) Untreated cells photographed with rhodamine and fluorescein optics, respectively. (Lower left and right) BFA-treated cells photographed with rhodamine and fluorescein optics, respectively. Bar = 10  $\mu\text{m}$ .

sized TCR subunits assemble rapidly into pentameric ( $\alpha\beta\gamma\delta\epsilon$ ) and heptameric ( $\alpha\beta\gamma\delta\epsilon\zeta_2$ ) complexes in the ER and are then transported through the Golgi apparatus either to lysosomes (i.e., pentamers) or the cell surface (i.e., heptamers) (Minami et al., 1987; Sussman et al., 1988). While BFA has no effect on the assembly of TCR subunits into multimeric complexes (Lippincott-Schwartz, unpublished data), the drug has a dramatic effect on the ultimate fate and subcellular distribution of these complexes, as described below.

#### Retention of TCR Chains in the ER of BFA-Treated T Cells

Biochemical data from several research groups have suggested that BFA, a lipophilic compound with the structure of a heterocyclic lactone (Harri et al., 1963), inhibits transport of proteins from the ER to the Golgi system (Misumi et al., 1986; Takatsuki and Tamura, 1985; Oda et al., 1987). To identify the site of blockade by BFA on intracellular transport of TCR chains in T cells, we used a morphological approach. Murine T cell hybridoma cells (2B4) were treated with or without BFA (10  $\mu\text{g/ml}$ ) (Sandoz Co., Switzerland) for various time periods and the resulting intracellular distribution of TCR chains was then examined by immunofluorescence and immunoelectron microscopy.

As shown by immunofluorescence (Figure 1, upper left), TCRs in control 2B4 cells detected with an anti- $\epsilon$  antibody were distributed primarily in large, phase-dense vesicles identified as lysosomes by electron microscopy (see below). Very faint staining of the plasma membrane was also observed. Double labeling of the same cells with antibodies to ER-resident proteins (Figure 1, upper right) showed virtually no coincidence between the two markers. These results on the subcellular distribution of TCR  $\epsilon$  chains are consistent with previous studies from our laboratory in

which the majority of TCR chains were observed to assemble into partial pentameric complexes (i.e.,  $\alpha\beta\gamma\delta\epsilon$ ) that were rapidly transported out of the ER, through the Golgi complex, and into lysosomes where they were degraded. Only the small subset of TCR complexes that were assembled into complete heptameric complexes were targeted to the cell surface (Minami et al., 1987; Sussman et al., 1988).

Addition of 10  $\mu\text{g/ml}$  BFA to the culture medium caused a dramatic change in the overall cellular distribution of TCRs in 2B4 cells. Three hours after the addition of the antibiotic, immunofluorescence staining of lysosomes by anti- $\epsilon$  antibody was no longer detectable (Figure 1, lower left). Instead, a fine reticular staining pattern along the nuclear membrane and throughout the cytoplasm was observed. Co-staining with antibodies to ER-resident proteins (Figure 1, lower right) revealed almost complete coincidence between the two labeling patterns. The reticular staining pattern by anti- $\epsilon$  antibodies was greatly reduced when the protein synthesis inhibitor cycloheximide was included in the medium (not shown). These results suggested that BFA treatment caused newly synthesized  $\epsilon$ -containing TCRs to be retained in the ER. Loss of staining by anti- $\epsilon$  antibodies from lysosomal structures during BFA treatment presumably was due to lysosomal degradation of the  $\epsilon$  chains since BFA reportedly has no inhibitory effect on lysosomal degradation (Misumi et al., 1986).

Morphological results similar to those of Figure 1 were obtained using antibodies to the TCR  $\alpha$  chain, indicating that transport of the TCR  $\alpha$  chain out of the ER was also blocked by BFA treatment (data not shown). That different TCR subunits are affected similarly by BFA is expected since the complete TCR complex ( $\alpha\beta\gamma\delta\epsilon\zeta_2$ ) assembles normally in BFA-treated cells. Thus a change in the subcellular distribution of one receptor chain, detected by an

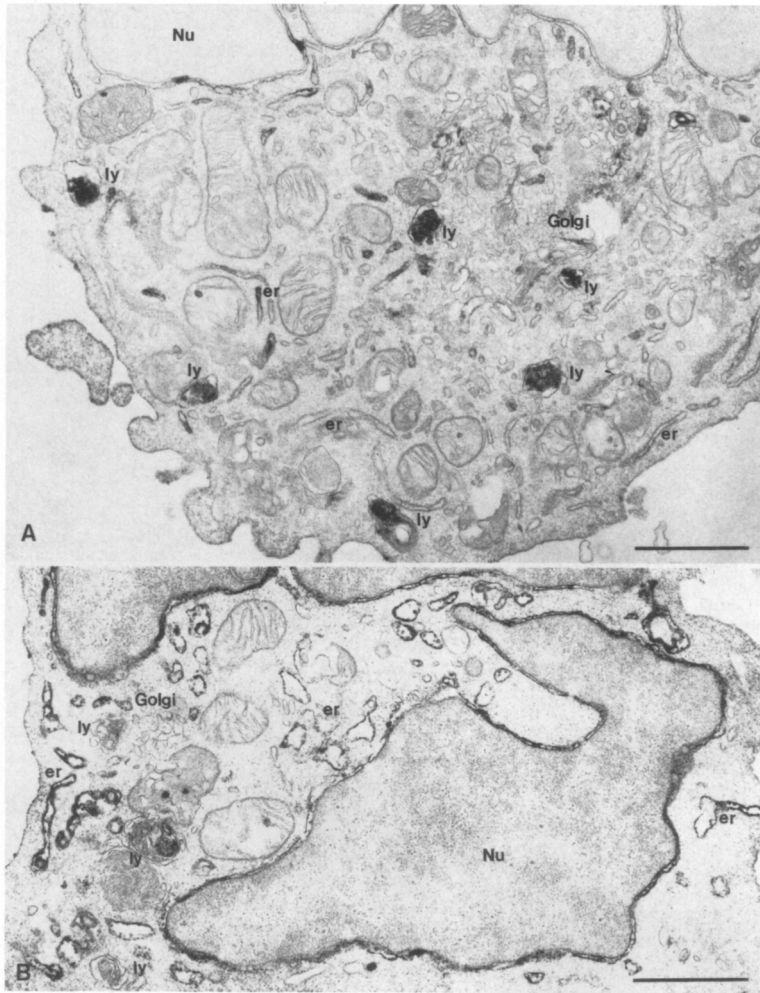


Figure 2. Effect of BFA on the Ultrastructural Distribution of TCR  $\epsilon$  Chains in T Cells

2B4 cells were incubated for 3 hr in the presence or absence of BFA (10  $\mu\text{g/ml}$ ) at 37°C. The cells were fixed, permeabilized with saponin, and labeled with anti- $\epsilon$  antibodies followed by HRP-conjugated goat anti-hamster IgG. After reaction with diaminobenzidine hydrochloride,  $\text{H}_2\text{O}_2$ , the cells were prepared for electron microscopy.

(A) Cytoplasm of untreated cells showing the distribution of HRP reaction product primarily in lysosomes. Faint labeling of the plasma membrane, Golgi apparatus, and ER (including the nuclear membrane) was also detected. Bar = 1  $\mu\text{m}$ .

(B) HRP-positive structures in BFA-treated cells. Intense labeling of ER cisternae and the nuclear envelope was observed. In contrast to (A), very little or no labeling of lysosomes, the Golgi apparatus, or plasma membrane was detected. Bar = 1  $\mu\text{m}$ . Abbreviations: Nu = nucleus; er = endoplasmic reticulum; ly = lysosomes.

antibody specific for this chain, reflects the distribution of other TCR chains in the assembled complex.

The effect of BFA on the subcellular distribution of  $\epsilon$ -containing TCRs was confirmed at the ultrastructural level using immunoperoxidase electron microscopy. In control cells (Figure 2A), horseradish peroxidase (HRP) staining was observed primarily in lysosomes and the Golgi complex. Slight staining of the plasma membrane and regions of the ER and nuclear membrane was also detected. After BFA treatment very little or no HRP staining was associated with lysosomes, the Golgi apparatus, or the cell surface. Instead, heavy staining was detected along ribosome-studded ER cisternae, in the nuclear membrane, and along vesicular and reticular ER, consistent with a block in transport of  $\epsilon$ -containing TCRs out of the ER. The morphology of the ER and Golgi complex was affected by the BFA treatment. ER cisternae were variably swollen, and Golgi stacks were no longer clearly visible. The morphology of other intracellular organelles, however, including lysosomes, mitochondria, and the nucleus, did not change as a result of the BFA treatment.

#### Processing of TCR Oligosaccharide Chains by Cis/Medial Golgi Enzymes in BFA-Treated Cells

If BFA inhibits transport of TCRs out of the ER, then one would predict that the oligosaccharide side chains of the receptor glycoprotein subunits would not be processed by Golgi-specific enzymes. This initially appeared to be the case, since the  $\alpha$  chains in pulse-chased 2B4 cells were not processed to the mature molecular mass of 42 kd observed in control cells (Figure 3A, top). Indeed, examination of the effect of neuraminidase on the electrophoretic mobility of  $\alpha$  in BFA-treated cells (Figure 3B) revealed that 1.5 hr after the biosynthetic pulse, these chains had not been modified with sialic acid, which requires transport to the *trans* Golgi compartment (Kornfeld and Kornfeld, 1985), in contrast to control cells. Nevertheless, when  $\alpha$  chain immunoprecipitates from control and BFA-treated cells were subjected to endoglycosidase H (endo H) digestion to compare their N-linked carbohydrate structures, rapid acquisition of endo H resistance by the  $\alpha$  chains in BFA-treated cells, surprisingly, was observed. Endo H cleaves "high-mannose" but not "complex"-type

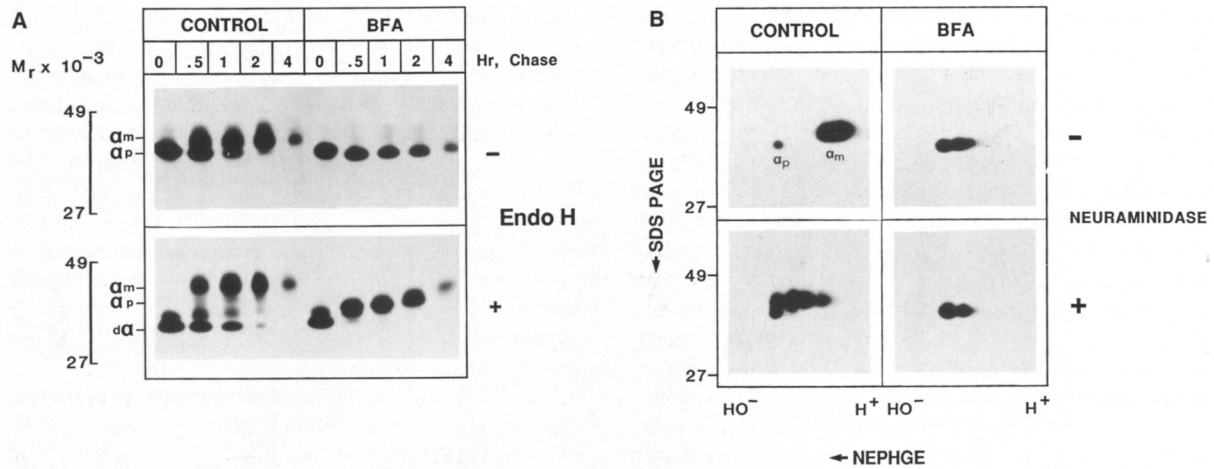


Figure 3. Processing of Newly Synthesized TCR  $\alpha$  Chains in BFA-Treated 2B4 Cells

(A) 2B4 cells were treated with or without BFA for 30 min prior to pulse-labeling with [<sup>35</sup>S]methionine for 10 min and chase in complete medium for the indicated times at 37°C. Cells were detergent solubilized, and  $\alpha$  chains were immunoprecipitated with anti- $\alpha$  antibodies. Immunoprecipitates were divided in half and treated with (lower) or without (upper) endo H before analysis by SDS-PAGE under reducing conditions. The positions of the precursor ( $\alpha_p$ ), mature ( $\alpha_m$ ), and deglycosylated ( $\alpha_d$ ) forms of the  $\alpha$  chain are marked. No effect on the rate of synthesis of TCR chains by BFA was detected.

(B) 2B4 cells were treated with or without BFA for 30 min, pulse-labeled for 10 min, and then chased in complete medium for 1.5 hr at 37°C. The chased cells were detergent solubilized, immunoprecipitated with anti- $\alpha$  antibodies, boiled for 5 min in 0.1 M sodium phosphate buffer (pH 6.1) containing 50 mM EDTA and 1% NP-40, and then incubated for 1 hr at 37°C in the presence (+) or absence (-) of 5 mU of neuraminidase (Calbiochem-Behring Corp., La Jolla, CA). Samples were analyzed by NEPHGE/SDS-PAGE under reducing conditions. Cleavage by neuraminidase of sialic acid residues on the mature  $\alpha$  oligosaccharide chains of control cells resulted in a shift in migration of these chains from an acidic to basic pH. No comparable shift in migration of the chains from BFA-treated cells was observed, indicating that these chains were not modified with sialic acid.  $M_r$  values ( $\times 10^{-3}$ ) are indicated at left.

oligosaccharide chains from proteins (Tarentino and Maley, 1974). Conversion from high-mannose to complex oligosaccharides occurs in the medial Golgi through the sequential activity of N-acetylglucosaminyltransferase and mannosidase II (Kornfeld and Kornfeld, 1985). As shown in Figure 3A (bottom), newly synthesized TCR  $\alpha$  chains began to show endo H resistance within 30 min of biosynthesis in BFA-treated cells. By 4 hr of chase, all of the  $\alpha$  oligosaccharide chains in the BFA-treated cells had become completely resistant to endo H, just as in the control cells. Note that the  $\alpha$  chain is progressively lost in both control and BFA-treated cells. In the former the  $\alpha$  chains are being transported to and degraded in lysosomes (Lip-

pincott-Schwartz et al., 1988). In the latter case the  $\alpha$  chains are subjected to ER degradation, which BFA does not inhibit (Lippincott-Schwartz et al., unpublished data). The  $\gamma$  chain of the TCR, as well, which also contains N-linked carbohydrate residues, showed evidence of processing by *cis*/medial Golgi enzymes in the BFA-treated cells. Roughly 50% of the  $\gamma$  chain carbohydrates became endo H resistant after 4 hr of chase (see below).

To test whether the acquisition of endo H resistance by the TCR oligosaccharide chains was due to processing by Golgi-specific enzymes and not merely to prolonged exposure of these chains to resident ER mannosidases, we examined the effect of swainsonine on these oligosaccha-

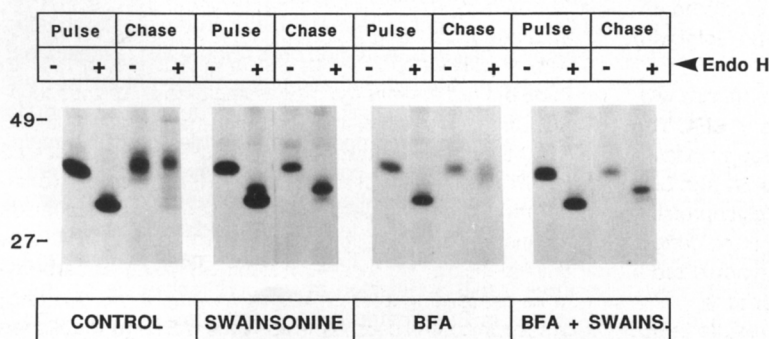


Figure 4. Effect of Swainsonine on the Acquisition of Endo H Resistance of TCR  $\alpha$  Glycoproteins in BFA-Treated Cells

2B4 cells were preincubated for 1 hr with no treatment (control), in the presence of swainsonine (10  $\mu$ g/ml) (swainsonine), with BFA (10  $\mu$ g/ml) (BFA), or with BFA plus swainsonine (BFA + swains.). The cells were then pulse-labeled with [<sup>35</sup>S]methionine for 10 min and chased for 4 hr in the same medium used for preincubation. The cells were detergent solubilized, immunoprecipitated with anti- $\alpha$  antibodies, and treated with or without endo H before analysis by SDS-PAGE under reducing conditions.  $M_r$  values ( $\times 10^{-3}$ ) are indicated at left.

ride processing events. Swainsonine specifically inhibits the action of Golgi mannosidase II, preventing the removal of  $\alpha$ 1 $\rightarrow$ 3 and  $\alpha$ 1 $\rightarrow$ 6 mannosyl residues, which are necessary for the acquisition of endo H resistance (Elbein et al., 1981; Tulsiani et al., 1982). 2B4 cells were preincubated for 1 hr in medium containing either no additions, swainsonine, BFA, or BFA plus swainsonine. The cells were then metabolically pulse-labeled for 10 min and chased for 4 hr in the same medium used for preincubation. Immunoprecipitation and endo H digestion were then performed to analyze the N-linked carbohydrate structures on the TCR  $\alpha$  subunits. As shown in Figure 4, swainsonine blocked the appearance of endo H-resistant  $\alpha$  oligosaccharides in both control and BFA-treated cells. Thus, endo H-resistant forms of  $\alpha$  in BFA-treated cells arise because of the activity of Golgi mannosidase II and not resident ER mannosidases.

These results presented an apparent paradox. The TCR complex in 2B4 cells is normally capable of rapid and efficient transport out of the ER and into the Golgi apparatus. With BFA treatment, TCRs are localized almost exclusively to the ER as determined by morphological studies, suggesting that the receptor is never transported out of the ER in the presence of this drug. Biochemical studies, however, suggest passage of the receptor into the *cis* and medial Golgi during BFA treatment, since TCRs obtain the signature (i.e., endo H resistance) for proteins that have reached this compartment.

One resolution of this paradox would be if BFA allowed movement of TCRs to the medial Golgi but blocked transport to the *trans* Golgi, instead rerouting the TCR complexes back to the ER. If this process were very fast, TCRs would accumulate only in the ER, consistent with our morphological observations of BFA-treated 2B4 cells. To investigate this possibility, we turned to two T cell lines in which the TCR chains are not transported into the Golgi system. The 2B4 variant  $\Delta$ 2 synthesizes all of the TCR chains except  $\delta$  (Bonifacino et al., unpublished data), and the murine T cell tumor line BW5147 fails to synthesize  $\delta$  and  $\zeta$  chains (Bonifacino et al., 1988). In the absence of one or more of the subunits, all other TCR subunits in these cells fail to negotiate ER-to-Golgi transport. This is presumably due to their inability to oligomerize properly. TCR glycoproteins in these cells never become endo H resistant, and by morphological criteria reside only in the ER (see below). Since TCRs in these cells are normally never transported to the Golgi, acquisition of endo H resistance during BFA treatment would not be predicted under the hypothesis of efficient movement to the Golgi with rapid retrieval to the ER.

$\Delta$ 2 and BW5147 cells were therefore pulse-chased with [ $^{35}$ S]methionine in the presence or absence of BFA. The TCR glycoproteins  $\alpha$  and  $\gamma$  were then immunoprecipitated and treated with or without endo H (Figures 5A and 5B). In untreated cells, as expected, both of the glycoproteins remained endo H sensitive throughout the chase period. Surprisingly, in the BFA-treated cells newly synthesized  $\alpha$  and  $\gamma$  chains became endo H resistant over time.

As further evidence that BFA treatment results in the Golgi-like carbohydrate processing of proteins retained in

the ER, we examined a resident ER protein. ERp99 is a glycoprotein of  $M_r = 99,000$  uniquely found and retained within the ER. Its characterization by Green and colleagues demonstrates that it remains in the ER at all times after its synthesis, with no Golgi processing of its carbohydrate chains (Lewis et al., 1985). The predominant form of its N-linked carbohydrate is  $\text{Man}_8\text{GlcNAc}_2$ . 2B4 cells were pulse-labeled with [ $^{35}$ S]methionine for 10 min and then chased for 2 hr in the presence or absence of 10  $\mu\text{g/ml}$  BFA. After immunoprecipitation with an anti-peptide antibody, the precipitates were either mock-treated or digested with endo H before being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In control cells the protein was identically sensitive to endo H at the end of the pulse and after the 2 hr chase. In BFA-treated cells ERp99 was sensitive to endo H at the end of the pulse but showed no shift in  $M_r$  in response to endo H after the chase (Figure 5C). This demonstrated that BFA can cause processing of resident ER proteins by Golgi enzymes. Together, these results are inconsistent with the hypothesis that BFA acts solely by blocking transport of proteins from the medial to the *trans* Golgi, since proteins retained in the ER, as well as exportable proteins, become modified by Golgi enzymes in the presence of this drug.

An alternative explanation for BFA's effects would be that, by inhibiting all protein transport out of the ER, it allows newly synthesized Golgi enzymes like mannosidase II to accumulate in the ER. This could explain how ER-resident proteins become modified by otherwise Golgi-specific enzymes in the presence of BFA. To test this hypothesis we looked at the effect of cycloheximide, a protein synthesis inhibitor, on the extent of carbohydrate processing of TCR chains in BFA-treated cells. If acquisition of endo H resistance by TCR glycoproteins in BFA-treated cells were due solely to the activity of newly synthesized mannosidase II, which has become trapped in the ER by BFA, then blocking protein synthesis prior to BFA treatment should prevent this by allowing newly synthesized proteins (like mannosidase II) to clear from the ER.

Figure 6 shows the results from an experiment designed to test this possibility. Carbohydrate processing in BW5147 cells of the TCR  $\gamma$  chain (a glycoprotein retained in the ER in these cells) was studied. BW5147 cells were metabolically pulse-labeled for 10 min and then incubated for 3.5 hr at 37°C in complete medium alone, for 3.5 hr in medium containing BFA, or for 1.5 hr in medium containing cycloheximide followed by 2 hr in medium containing BFA plus cycloheximide.  $\gamma$  Chain glycoproteins were then immunoprecipitated at various times of chase and treated with or without endo H. As shown in Figure 6, cycloheximide did not prevent the processing of the  $\gamma$  oligosaccharide chains to endo H-resistant forms in cells treated with BFA, even though lower quantities of newly synthesized mannosidase II should occur in the ER of these cells compared with control cells; in the latter,  $\gamma$  oligosaccharide chains remain endo H sensitive. Cycloheximide did slow the kinetics of  $\gamma$  processing 2-fold. Slowing of carbohydrate processing after prolonged treatment with this drug is seen in control cells in the absence of BFA (unpublished observations). Thus, newly synthesized mannosidase II

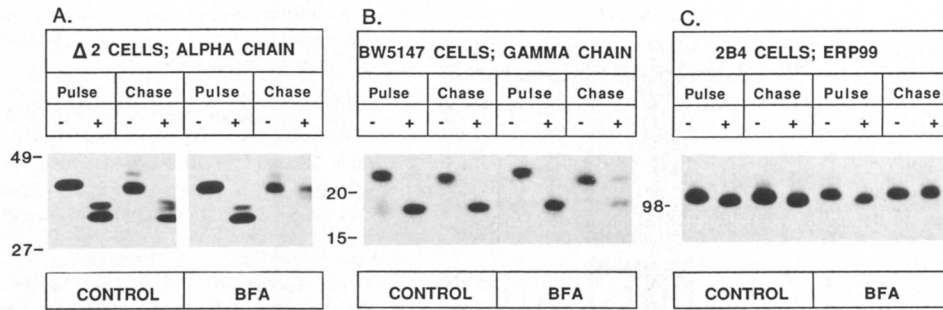


Figure 5. Acquisition of Endo H Resistance by Resident ER Glycoproteins During BFA Treatment

$\Delta 2$ , BW5147, or 2B4 cells were preincubated for 1 hr in the presence or absence of 10  $\mu$ M BFA prior to pulse-labeling with [<sup>35</sup>S]methionine for 10 min and chasing in preincubation medium for 4 hr. The cells were detergent solubilized and immunoprecipitated with anti- $\alpha$  antibodies (A), with anti- $\gamma$  antibodies (B), or with anti-ERP99 antibodies (C). Immunoprecipitates were treated with (+) or without (-) endo H before analysis by SDS-PAGE under reducing conditions. M<sub>r</sub> values ( $\times 10^{-3}$ ) are indicated at the left side of each panel.

trapped in the ER of BFA-treated cells is unlikely to account for the acquisition of endo H resistance by TCR oligosaccharide chains in these cells.

#### Rapid Redistribution of *Cis*/Medial Golgi Enzymes to the ER in BFA-Treated Cells

A third, more provocative explanation for how glycoproteins retained in the ER acquire endo H resistance in the presence of BFA would be that the responsible Golgi en-

zymes themselves redistribute from the Golgi apparatus to the ER. To test for this possibility, we examined the morphological distribution of the *cis*/medial Golgi marker mannosidase II (Dunphy and Rothman, 1983; Farquhar, 1985; Kornfeld and Kornfeld, 1985), using antibodies to this enzyme in cells treated with or without BFA. As shown in Figure 7 (upper left), immunofluorescence staining for mannosidase II in permeabilized, normal rat kidney (NRK) cells revealed intense labeling of large, perinuclear structures characteristic of the Golgi apparatus. After 30 min of treatment with BFA, however, the distribution of mannosidase II had changed dramatically. The entire population of the Golgi enzyme had dispersed into a fine, punctate reticulum distributed throughout the cytoplasm (Figure 7, lower left).

Immunoperoxidase electron microscopy was performed to identify at the ultrastructural level the intracellular compartment(s) into which mannosidase II had redistributed during the BFA treatment. Rat basophilic leukemia (RBL) cells were used for this purpose since staining by mannosidase II antibodies was greater in these cells than in other cells tested. However, the pattern of staining and redistribution was essentially identical in T cells (see below), fibroblasts, and RBL cells. As shown in Figure 8A, the HRP reaction product in control RBL cells was localized almost entirely to *cis* and medial Golgi cisternae. After BFA treatment (Figure 8B) virtually no HRP-positive Golgi cisternae could be detected in these cells. Instead, HRP staining was localized within the nuclear membrane, along ribosome-studded ER cisternae, and within vesicular and reticular elements of the ER. No labeling of lysosomes, mitochondria, multivesicular bodies, or the plasma membrane was observed in the BFA-treated cells. When cells were pretreated with cycloheximide to block new protein synthesis, the same morphological redistribution of mannosidase II was observed. These results indicate that, in the presence of BFA, mannosidase II is no longer localized in Golgi cisternae but has redistributed dramatically to the ER.

The distribution of a different Golgi marker—mannosidase IA, primarily a *cis* Golgi component (Dunphy et al., 1983; Snider and Rogers, 1986; Kornfeld and Kornfeld,

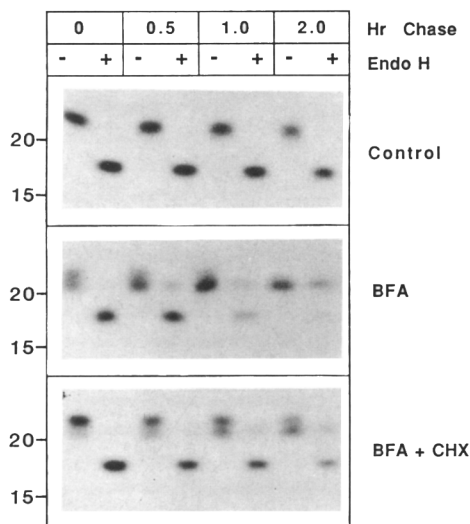
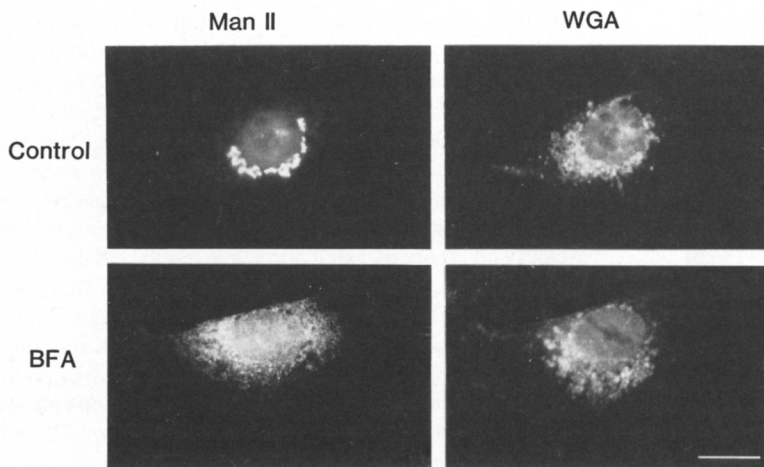


Figure 6. Effect of Cycloheximide on the Acquisition of Endo H Resistance by ER-Retained TCR  $\gamma$  Chains in BFA-Treated Cells

BW5147 cells were pulse-labeled with [<sup>35</sup>S]methionine for 10 min and then incubated for 3.5 hr at 37°C in complete medium alone (control), for 3.5 hr in complete medium containing BFA (10  $\mu$ M/ml) (BFA), or for 1.5 hr in complete medium containing cycloheximide (10  $\mu$ M/ml; Sigma) and then 2 hr in medium containing BFA plus cycloheximide (BFA + CHX). Treatment of cells with 10  $\mu$ M/ml cycloheximide resulted in more than 95% inhibition of protein synthesis. The indicated time points for chase were started 1.5 hr after the end of the pulse. At these times the cells were detergent solubilized and immunoprecipitated with anti- $\gamma$  antibodies. Immunoprecipitates were then treated with (+) or without (-) endo H before analysis by SDS-PAGE under reducing conditions. M<sub>r</sub> values ( $\times 10^{-3}$ ) are indicated at left.

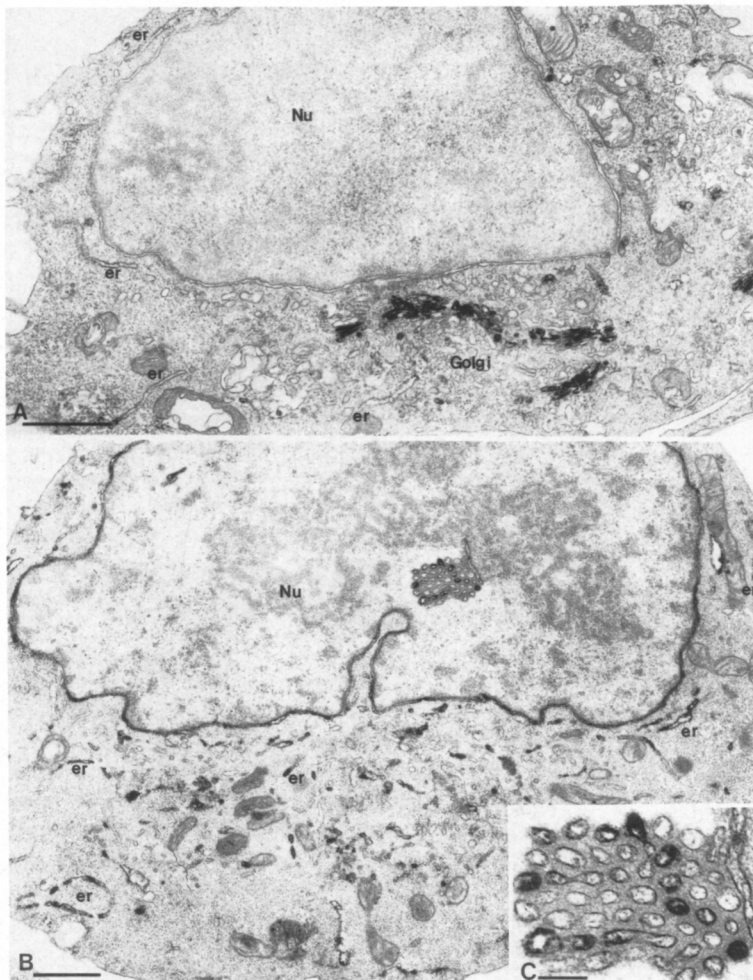


**Figure 7. Immunofluorescence Localization of Mannosidase II and *Trans* Golgi Proteins in BFA-Treated and Untreated Cells**

NRK cells were incubated for 30 min in the presence or absence of BFA (10  $\mu\text{g/ml}$ ) at 37°C. The cells were then fixed and incubated with 50  $\mu\text{g/ml}$  WGA (E-Y Laboratories) to block surface lectin binding sites. After washing to remove unbound WGA, the cells were incubated with FITC-conjugated WGA (E-Y Laboratories) and anti-mannosidase II antibodies in the presence of 0.5% saponin. The cells were washed again and incubated with rhodamine-labeled goat anti-rabbit IgG to label the specifically bound anti-mannosidase II antibodies. After a final wash, the cells were prepared for microscopy. (Upper left and right) Control cell photographed in rhodamine (left) and fluorescein (right) optics to identify mannosidase II and WGA binding, respectively. (Lower left and right) BFA-treated cell photographed in rhodamine (left) and fluorescein (right) optics. Bar = 10  $\mu\text{m}$ .

1985)—was also examined to determine whether other components of the *cis*/medial Golgi were also redistributed to the ER upon exposure to BFA. Immunofluores-

cence staining revealed that the mannosidase IA distribution changed from a predominantly Golgi-like staining pattern to a reticular, ER labeling pattern upon BFA treat-



**Figure 8. Effect of BFA on the Ultrastructural Distribution of Mannosidase II**

RBL cells were incubated for 4 hr in the presence or absence of BFA (10  $\mu\text{g/ml}$ ) at 37°C. The cells were fixed, permeabilized with saponin, and labeled with rabbit anti-mannosidase II antibodies followed by HRP-conjugated goat anti-rabbit IgG. After reaction with diaminobenzidine hydrochloride,  $\text{H}_2\text{O}_2$ , the cells were prepared for electron microscopy.

(A) Cytoplasm of control cells showing the distribution of HRP reaction product localized almost completely to *cis*/medial Golgi cisternae. Bar = 1  $\mu\text{m}$ .

(B) Cytoplasm of BFA-treated cells showing HRP labeling of ER cisternae and the nuclear membrane. No labeling of lysosomes, mitochondria, or the plasma membrane was detected. Golgi stacks were disrupted by the BFA treatment and showed no HRP staining. Bar = 1  $\mu\text{m}$ . (Inset) High-magnification view of HRP-labeled, sinusoidal smooth ER juxtapsed to the nucleus. Bar = 0.2  $\mu\text{m}$ . Abbreviations: Nu = nucleus; er = endoplasmic reticulum.



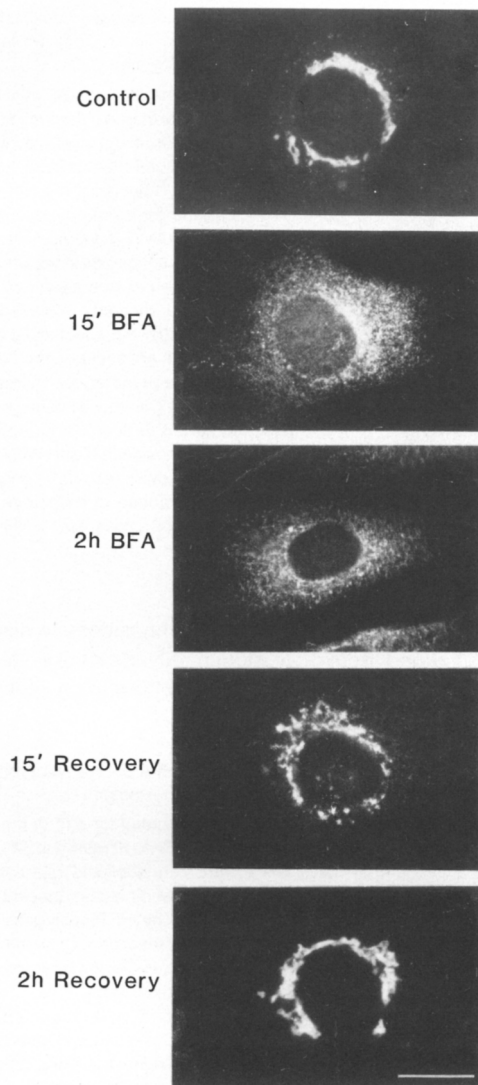


Figure 9. Time Course of BFA-Induced Disruption and Recovery of the Golgi Apparatus

NRK cells were incubated with BFA (10  $\mu$ g/ml) for 0 (control), 15 min, or 2 hr. Alternatively, NRK cells were incubated for 2 hr with BFA and then chased in fresh, untreated medium for 15 min or 2 hr to allow recovery from BFA treatment. The cells were then fixed, permeabilized with saponin, and labeled with anti-mannosidase II antibodies. After washing to remove unbound IgG, cells were incubated with rhodamine-labeled goat anti-rabbit IgG to label the specifically bound anti-mannosidase II antibodies. After a final wash the cells were prepared for microscopy and photographed in rhodamine optics. Bar = 10  $\mu$ m.

ment (not shown). These results, combined with the above ultrastructural observations, raised the possibility that the *cis*/medial Golgi complex itself becomes a part of the ER during BFA treatment.

#### Structural Segregation of *Cis*/Medial from *Trans* Golgi in BFA-Treated Cells

In contrast to the distribution of mannosidases IA and II, the distribution of *trans* Golgi glycoproteins, as detected

by immunofluorescence staining with wheat germ agglutinin (WGA), did not change significantly during BFA treatment. WGA binds to clustered terminal N-acetylneuraminic acid residues as well as N-acetylglucosamine-containing oligosaccharide chains on proteins (Tartakoff and Vassalli, 1983). As a result, it labels cisternae along the distal face of the Golgi stack, associated vesicles, and the cell surface (Virtanen et al., 1980). Co-staining of NRK cells with mannosidase II and fluorescently labeled WGA (Figure 7) revealed a mixed staining pattern of Golgi and associated vesicles by WGA, in contrast to the mannosidase II staining of the *cis*/medial Golgi. Plasma membrane staining by fluorescent WGA was blocked by preincubation of the intact cells with unlabeled WGA. Upon BFA treatment little or no change in the staining pattern for WGA occurred, in contrast to that for mannosidase II. Morphologic studies using a monoclonal antibody directed against a resident integral membrane protein of the *trans* Golgi (Yuan et al., 1987) confirmed the WGA results and demonstrated that the *trans* Golgi does not redistribute with the *cis*/medial Golgi in response to BFA (data not shown). These results suggested that BFA exerts a profound effect on the structure of the Golgi apparatus, effectively causing a spatial segregation of *cis*/medial components (which redistribute to the ER) from the *trans* Golgi (which shows no localization to the ER).

#### Rapid Reassembly of the Golgi after Removal of BFA

The redistribution of mannosidase II to the ER when cells were treated with BFA was rapid. This is depicted morphologically in Figure 9. Within 15 min of BFA treatment, virtually the entire population of mannosidase II in NRK fibroblasts had redistributed from a perinuclear Golgi staining pattern into a punctate, reticular ER staining pattern. No further change was observed with longer incubations in the presence of BFA. Reassembly of the Golgi apparatus into the tightly organized perinuclear structure found in control cells occurred rapidly upon removal of BFA from cells. As shown in Figure 9, mannosidase II had almost completely redistributed from a diffuse reticular/vesicular pattern (characteristic of the ER) to a central perinuclear structure within 15 min of removal of BFA. By 2 hr the staining pattern for mannosidase II showed large perinuclear structures and was indistinguishable from that in control cells.

#### Selective Intracellular Transport of TCR Chains Is Restored upon Removal of BFA

To test whether the inhibitory effects of BFA on the intracellular transport and sorting of TCR chains could be fully reversed upon removal of this lipophilic drug, we examined the subcellular distribution of the TCR with anti- $\epsilon$  chain antibodies before, during, and after BFA treatment. We used 2B4 cells and the tumor line BW5147 for this purpose, since in the former cell type TCR complexes are normally rapidly transported out of the ER and into the Golgi apparatus, while in the latter these complexes normally fail to leave the ER. In these experiments cycloheximide was used to block new protein synthesis. Thus, the immu-

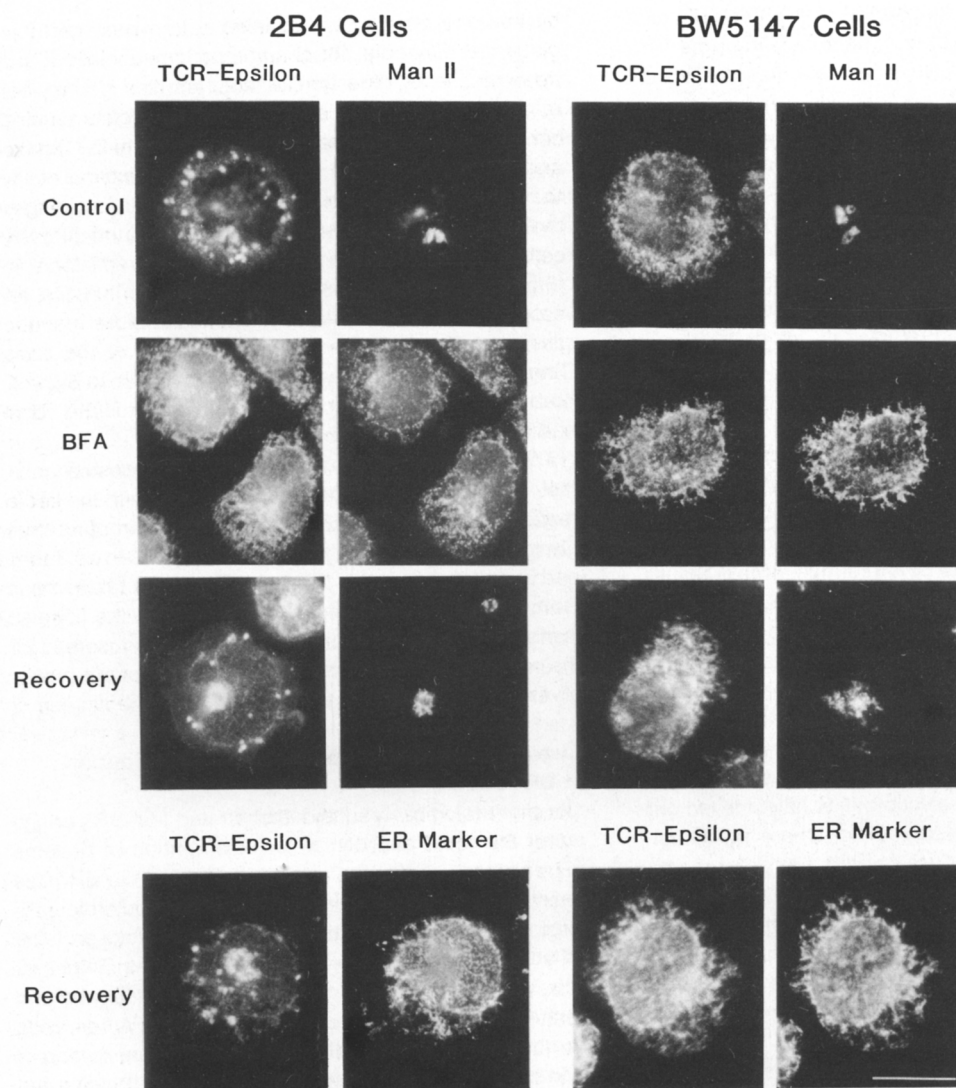


Figure 10. Sorting of Resident ER Proteins From Exportable Proteins upon Removal of BFA

2B4 and BW5147 cells were treated with BFA for 4 hr (BFA), treated with BFA for 4 hr and then reincubated in fresh medium containing 10  $\mu$ g/ml cycloheximide for 1.5 hr (recovery), or left untreated (control). The cells were then fixed, permeabilized with saponin, and double labeled with anti- $\epsilon$  antibodies plus antibodies against ER proteins, or with anti- $\epsilon$  antibodies plus antibodies against mannosidase II. After washing to remove unbound IgG, cells were incubated with rhodamine-labeled goat anti-hamster IgG to label the specifically bound  $\epsilon$  antibodies and with fluorescein-labeled goat anti-rabbit IgG to label specifically bound ER-directed or anti-mannosidase II antibodies. After a final wash the cells were prepared for microscopy. Bar = 10  $\mu$ m.

nofluorescence changes after removal of BFA reflected true redistribution of TCR proteins.

Immunofluorescence staining of the TCR  $\epsilon$  chain in 2B4 cells (Figure 10, left) revealed that these chains resided in the ER during BFA treatment, but once BFA was removed the chains redistributed throughout the secretory pathway to a steady state indistinguishable from the distribution in control cells. In both the recovered and control 2B4 cells, TCR  $\epsilon$  chains were localized predominantly in large vesicles corresponding to lysosomes and Golgi, in contrast to the fine reticular-vesicular network in which they were found during BFA treatment. Double labeling of the cells with antibodies to mannosidase II revealed the same

redistribution of the Golgi enzyme to the ER upon BFA treatment as was observed in NRK and RBL cells. Likewise, removal of BFA resulted in transport of mannosidase II out of the ER into Golgi cisternae. Double labeling of the recovered cells with antibodies against the TCR  $\epsilon$  chain and ER-resident proteins demonstrated that ER-resident proteins with which TCR  $\epsilon$  chains and mannosidase II had mixed during BFA treatment remained in the ER. Thus, removal of BFA resulted in the rapid and efficient sorting of membrane components from the ER.

Immunofluorescence staining of TCR  $\epsilon$  chains in BW 5147 cells (Figure 10, right) revealed essentially no change in the distribution of TCR chains before, during, or after

BFA treatment. At all times staining of the TCR  $\epsilon$  chains had a reticular-network pattern characteristic of the ER. Double labeling of these cells with antibodies to ER-resident proteins (bottom right two panels) confirmed the localization of  $\epsilon$  to the ER. The BFA-induced redistribution of mannosidase II and its recovery upon removal of the drug were identical in BW5147 and 2B4 cells. Altogether, these results suggest that BFA has no permanent effect on the mechanisms for intracellular trafficking of proteins in cells, since sorting of resident ER proteins (i.e.,  $\epsilon$  chains in BW5147 cells) from exportable proteins (i.e.,  $\epsilon$  chains in 2B4 cells) is restored upon removal of this drug.

### Discussion

The study reported here grew out of an attempt to resolve an apparently paradoxical set of observations on the effects of BFA on the fate of glycoprotein subunits of the TCR. Both immunofluorescence and immunoelectron microscopy demonstrated that BFA treatment of cells results in the accumulation of TCR chains in the ER, suggesting that BFA blocks protein transport out of the ER. In contrast, biochemical analysis of the carbohydrate side chains of the  $\alpha$  and  $\gamma$  TCR subunits in BFA-treated cells indicated they are processed in a manner consistent with movement into the medial Golgi. In particular, both of these subunits became resistant to cleavage by the enzyme endo H. This normally occurs only after glycoproteins have moved out of the ER and into the medial Golgi where the Golgi-specific enzymes mannosidase II and N-acetylglucosaminyltransferase I convert the oligosaccharide side chains to an endo H-resistant, complex form. The ability of swainsonine, a specific inhibitor of Golgi mannosidase II, to block the acquisition of endo H resistance in the BFA-treated cells supported the conclusion that we were observing processing by Golgi enzymes.

To resolve the apparent conflict in these experimental results, we explored three possible explanations for the effects of BFA on the fate of TCR glycoproteins: one, that BFA does not block transport of the glycoproteins out of the ER, but instead rapidly reroutes them back to the ER after they have reached the medial Golgi; two, that BFA acts as a general block to protein transport out of the ER, resulting in the accumulation and activity of newly synthesized Golgi enzymes in the ER; and three, that BFA induces the redistribution of Golgi enzymes to the ER while preventing transport of proteins out. Our results strongly favored the third hypothesis—that Golgi enzymes themselves redistribute from the Golgi apparatus to the ER during BFA treatment. The characteristics of this redistribution phenomenon suggested that BFA may interfere with a normal recycling pathway between the ER and *cis*/medial Golgi, efficiently blocking the cycling pathway out of but not back to the ER.

### Evidence for the Redistribution of *Cis*/Medial Golgi Enzymes to the ER of BFA-Treated Cells

When we examined the fate of Golgi-resident proteins in cells treated with BFA, an explanation for our original data emerged. Within minutes of the addition of BFA, immuno-

fluorescence microscopy revealed a dramatic redistribution of the *cis*/medial Golgi markers mannosidase II and mannosidase IA. The typical, concentrated perinuclear Golgi pattern was replaced by a diffuse, punctate staining that coincided with the reticular pattern of the ER. Immunoelectron microscopy confirmed the impression of the fluorescence patterns and demonstrated clear staining of the ER. The *cis*/medial Golgi proteins were found diffusely throughout the ER, including the nuclear envelope. In contrast, staining of *trans* Golgi proteins with WGA revealed that these proteins did not redistribute with the *cis*/medial Golgi markers. The distribution of the *trans* Golgi proteins only slowly changed in response to BFA addition, gradually appearing in vesicular structures that had no spatial relationship to the ER.

These results indicated that the *cis*, and possibly medial, Golgi rapidly disassemble and merge with the ER in response to BFA. The ER retains its structure, and thus glycoproteins retained in the ER are processed as if they had been transported to the Golgi. The fact that the *trans* Golgi does not follow the *cis*/medial Golgi to the ER also explained why no addition of sialic acid was observed for the proteins retained in the ER. A summary of our results on the effects of BFA is presented in Table 1.

### Characteristics of Golgi Enzyme Redistribution in BFA-Treated Cells

Our morphological results demonstrated that BFA brings about the rapid and complete redistribution of proteins normally restricted to the *cis*/medial Golgi to the ER. This redistribution is specific in that *cis* and medial Golgi proteins redistribute only to the ER, and *trans* Golgi proteins do not follow the route taken by the rest of the Golgi system. The effect is rapid (on the order of 5 to 15 min) and reversible. The consequences of removing BFA from cells demonstrate graphically the rapid and efficient sorting of membrane components from the ER. Golgi proteins are rapidly removed from the ER into the newly reformed Golgi cisternae. ER-resident proteins with which they had been mixed remain in the ER. Likewise, TCR complexes rapidly recover their ability to be transported out of the ER, through the Golgi, and onto more distal target organelles. Yet the structural requirements for ER-to-Golgi transport are retained. Thus, in BW5147 cells the  $\gamma\epsilon$  complex, despite the Golgi processing of  $\gamma$  carbohydrate chains, remains trapped within the ER, even after removal of the BFA.

The presence of *cis*/medial Golgi enzymes in the ER may have different consequences for the oligosaccharide processing of different glycoproteins, depending on how active these enzymes are in the luminal environment of the ER. Thus, while the oligosaccharide chains of TCR  $\alpha$  and ERp99 are rapidly converted to endo H-resistant forms in BFA-treated cells, other glycoprotein oligosaccharide chains, including TCR  $\gamma$  and the vesicular stomatitis virus G protein (see Takatsuki and Tamura, 1985) require much longer incubation times with BFA for such processing events to be detected. In a recent study by Perkel et al. (1988), the effects of BFA on the processing of high-mannose oligosaccharide chains on glycoproteins

Table 1. Summary of the Effects of BFA on the Subcellular Distribution and Carbohydrate Processing of Proteins

Protein	Cell Type	Morphological Localization		Carbohydrate Processing (Endo H Sensitivity)	
		- BFA	+ BFA	- BFA	+ BFA
TCR $\alpha$	2B4	Golgi/Ly/PM	ER	resistant (+ SA)	resistant (- SA)
TCR $\alpha$	$\Delta 2$	ER	ER	sensitive	resistant
TCR $\gamma$	2B4	Golgi/Ly/PM	ER	resistant	resistant
TCR $\gamma$	BW5147	ER	ER	sensitive	resistant
TCR $\epsilon$	2B4	Golgi/Ly/PM	ER	O	O
TCR $\epsilon$	BW5147	ER	ER	O	O
ERp99	all tested	ER	ER	sensitive	resistant
Mannosidase II	all tested	Golgi	ER	NT	NT
Mannosidase IA	all tested	Golgi	ER	NT	NT

Abbreviations include: Ly = lysosomes; PM = plasma membrane; NT = not tested; SA = sialic acid; O = no oligosaccharide side chains. The changes in subcellular localization of proteins induced by BFA that are indicated here are derived from experiments with cells treated with BFA for more than 3 hr. With shorter incubations in the drug, TCR proteins in 2B4 cells were detected in lysosomes and the PM (in addition to the ER) due to the half-life of the receptor in these compartments. None of the proteins were observed in Golgi-like structures after either short- or long-term BFA treatment.

from ER subcellular fractions was examined. In cells treated with BFA, they observed an accumulation of high-mannose oligosaccharides containing 5–8 mannose residues instead of the 8 or 9 mannose residues found in control incubations.

#### Possible Mechanisms for BFA's Effects

As with other drugs that have been used to perturb intracellular protein transport, it is difficult to determine the exact site or sites of action of BFA and whether the drug has only a single mechanism of perturbing cellular function. Previous studies with BFA demonstrated that it primarily interferes with protein secretion; it has no noticeable effects on endocytosis, lysosomal degradation, or protein synthesis (Misumi et al., 1986). We feel that BFA is acting by interfering with a normal recycling pathway between the Golgi and the ER. We can picture two ways in which this could explain our observations. First, the *cis* Golgi may be constantly recycling back to the ER and then efficiently sorted out of the ER and returned to the Golgi. If BFA blocked the latter step, the Golgi would simply continue to recycle to the ER but remain there. Second, lipid-rich vesicles may be constantly recycling from the Golgi to the ER without resident Golgi proteins. This would require a specific retention mechanism for keeping proteins within the Golgi stack while allowing lipid to return to the ER. If BFA abrogated the Golgi retention mechanism, then the Golgi proteins could recycle with the lipid. In effect, the Golgi would drain back to the ER, utilizing an underlying vesicular recycling pathway. The failure, then, of proteins to leave the ER would be a consequence of the absence of a target organelle (i.e., the *cis* Golgi) for any vesicles leaving the ER. Rather, the ER itself (since, in the presence of BFA, it contains all of the components of the *cis* Golgi) would be the target for such vesicles.

#### Implications of Recycling from Golgi to ER

A recycling model for transport between the Golgi and ER provides a resolution to the problems of lipid flow between the ER and Golgi. The constant redistribution of lipid

through such an interlocking recycling system would allow the tremendous lipid flow out of the ER to be returned via a vesicular transport mechanism. This model would have a number of further implications. Based on previous studies, the rate of membrane flow through this recycling system would have to be rapid. Wieland et al. (1987) have measured the rate of membrane bulk flow out of the ER, estimating that the equivalent of at least half of the surface area of the ER is transported to the Golgi every 10 min. Assuming retrieval of this massive quantity of lipid occurs by recycling from the Golgi apparatus, these authors estimate that the average lipid in the Golgi would have a lifetime of about 1 to 2 min before it is recycled back to the ER. Our results demonstrating rapid kinetics for both movement of Golgi proteins into the ER in cells treated with BFA and their return to the Golgi upon removal of the drug are consistent with these estimates.

An implication of protein recycling between the *cis* Golgi and ER would be the transient mixing of Golgi with ER-retained proteins. Such mixing might cause gradual processing of the carbohydrate chains of long-lived ER proteins by Golgi enzymes. Two mechanisms might make the observations of such processing events difficult. Perhaps some characteristic of the site of mixing within either an intermediate compartment or within a region of the ER prevents the functioning of Golgi enzymes. Second, there might exist in the ER a repair mechanism that compensates for a low rate of Golgi processing (either by recycled or newly synthesized Golgi enzymes) of ER-resident proteins. Whether, and to what extent, Golgi processing events occur in the ER has not been extensively examined. One study failed to detect Man<sub>5</sub>GlcNAc<sub>2</sub> on ER-resident proteins, suggesting that they had not cycled through the *cis* Golgi (Brands et al., 1985). However, recent data of Pelham (1988) have demonstrated that an ER-retained protein is modified by a phosphotransferase believed to be localized to a post-ER, *cis* Golgi compartment.

Increasing attention has been focused on a vesicular compartment situated between the ER and the *cis* Golgi (Warren, 1987; Saraste and Kuismanen, 1984; Schweizer

et al., 1988). It is this compartment that is the site of the low-temperature block to ER-to-Golgi transport (Saraste et al., 1986). It also appears to be the site of budding of coronaviruses (Tooze et al., 1988). Perhaps the recycling from the Golgi occurs into this intermediate compartment in which ER and Golgi proteins might mix and be sorted. Such a compartment would be analogous to the endosome. If it proves to be truly analogous to the endosome, however, it is likely to be a complex organelle, both functionally and structurally.

Underlying any of the explanations for the effects of BFA is the apparent specific "affinity" of the *cis*/medial Golgi for the ER. We do not think that it is a coincidence that the *cis*/medial Golgi, into which ER components normally flow, is capable of returning to the ER. It is interesting that in this respect the *cis*/medial Golgi is completely dissociated from the *trans* Golgi. The *trans* Golgi has been implicated in recycling pathways with the endocytic organelle system. Perhaps the structural dissociation we observe is telling us that two supraorganellar systems exist in the secretory/endocytic pathways. One is composed of the ER, intermediate compartment, and *cis* and medial Golgi, while the second consists of the *trans* Golgi, *trans* Golgi network, endosomes, lysosomes, and plasma membrane. As with the endocytic pathway, the characterization of drugs that perturb the early trafficking events in the secretory pathway will be of great help in illuminating these critical cellular processes.

#### Experimental Procedures

##### Cells

The murine T cell hybridoma 2B4.11 expresses the complete seven-chain TCR (Samelson et al., 1985). It was generated by Hedrick et al. (1982) from a fusion of pigeon cytochrome c-primed lymph node cells to the murine BW5147 thymoma cell line. The murine thymoma cell line BW5147.3 fails to express the  $\delta$  and  $\zeta$  TCR chains (Bonifacino et al., 1988). It was obtained from the American Type Culture Collection, Rockville, MD. The murine T cell hybridoma  $\Delta 2$  is deficient in the expression of the TCR  $\delta$  chain. It was generated by Bonifacino et al. (unpublished data) by repetitive subcloning at limiting dilution of  $\delta$ - and  $\zeta$ -deficient variants of 2B4.11 cells, followed by transfection with TCR  $\zeta$  chain cDNA. All of the above T cell lines were grown in suspended cell culture as described in Samelson and Schwartz (1983). NRK and RBL cells were grown in RPMI 1640 with 10% fetal bovine serum.

##### Antibodies

The following antibodies were used to precipitate the TCR chains: anti- $\alpha$ , A2B4-2, monoclonal murine IgG2a that binds the  $\alpha$  chain expressed by 2B4 cells (Samelson et al., 1983); anti- $\gamma$ , 125, polyclonal antiserum raised in a rabbit immunized with purified  $\epsilon$  and  $\gamma$  chains; anti- $\epsilon$ , 500A2, monoclonal hamster IgG that binds to murine  $\epsilon$  chain (Leo et al., 1987). Rabbit IgG directed against Golgi mannosidase II (Moremen and Touster, 1985) was a kind gift of Dr. K. Moremen (M.I.T.). Rabbit IgG directed against Golgi mannosidase IA was a kind gift of Dr. Oscar Touster (Vanderbilt University). Rabbit polyclonal antibodies to ER-resident proteins (Louvard et al., 1982) were generously provided by Dr. D. Louvard (Pasteur Institute, Paris, France). Rabbit anti-peptide antibodies to ERp99 were kindly provided by Dr. Michael Green (St. Louis University). Rhodamine- and fluorescein-labeled goat anti-rabbit IgG, rhodamine- and fluorescein-labeled goat anti-hamster IgG, HRP-conjugated goat anti-hamster F(ab)<sub>2</sub>, and goat anti-rabbit F(ab)<sub>2</sub> were purchased from Cooper Biomedical (Malvern, PA).

##### Metabolic Labeling, Immunoprecipitation, and Electrophoresis

Cells were metabolically labeled with [<sup>35</sup>S]methionine as follows: Approximately  $2 \times 10^7$  cells per time point were preincubated for 5 min

at 37°C in methionine-free RPMI 1640 medium (Biofluids, Rockville, MD) containing 5% fetal bovine serum. The cells were labeled for 10 min at  $1 \times 10^7$  cells per ml with 0.25 mCi/ml [<sup>35</sup>S]methionine (Trans-<sup>35</sup>S-label, ICN, Irvine, CA). Following the labeling period the cells were resuspended in complete culture medium. At appropriate time intervals the chase was terminated by placing the cells on ice. Solubilization and immunoprecipitation using antibody adsorbed to protein A-agarose (Bethesda Research Laboratories) were performed as previously described (Lippincott-Schwartz et al., 1988). Immunoprecipitated proteins were analyzed by SDS-PAGE on one- or two-dimensional polyacrylamide gels (13%) using the buffer systems of Laemmli as described in Samelson et al. (1985). Two-dimensional nonequilibrium pH-gradient electrophoresis (NEPHGE)/SDS-PAGE was performed under reducing conditions as described in Samelson et al. (1985).

##### Immunofluorescence Microscopy

Cells were either grown on 12 mm round coverslips or attached to concanavalin A-coated coverslips as described below in "Other Procedures." After appropriate treatments the cells were fixed in 2% formaldehyde, 0.1 M sodium phosphate (pH 7.4), for 10 min at 25°C. The cells were next permeabilized with 0.15% saponin in PBS, 0.1% BSA, for 15 min and then incubated in antibody-containing medium. The cells were then washed in PBS, 0.1% BSA, incubated with fluorescently labeled second antibody, and washed again. Antibody incubations were for 1.5 hr at 25°C. Coverslips were mounted on glass slides in Fluoromount G (Southern Biotechnology) and viewed with a Zeiss ICM405 inverted microscope equipped with barrier filters to prevent crossover of fluorescein and rhodamine fluorescence.

##### Immunoperoxidase Electron Microscopy

Cells grown on glass coverslips were treated with or without BFA and then fixed with 2% formaldehyde containing lysine and periodate (McLean and Nakane, 1974), permeabilized with saponin, and then incubated with anti- $\epsilon$  TCR antibodies or anti-mannosidase II antibodies. After washing to remove unbound IgG, the cells were incubated with HRP-conjugated goat anti-hamster or goat anti-rabbit IgG, incubated with diaminobenzidine hydrochloride, H<sub>2</sub>O<sub>2</sub>, and prepared for electron microscopy, all as described in Yuan et al. (1987).

##### Other Procedures

Digestion with endo H (Genzyme, Boston, MA) was performed by incubating the immunoprecipitates for 16 hr at 37°C with 5 mU of endo H in 50  $\mu$ l of 0.1 M sodium phosphate buffer (pH 6.1) containing 0.1% Triton X-100, 0.03% SDS, and 20 mM EDTA.

To attach suspended cells (including 2B4 and BW5147 cells) to coverslips for immunofluorescence microscopy, the cells were washed twice at 4°C in HEBs buffer (containing 20 mM HEPES, 140 mM NaCl, 0.5 mM KCl, 3 mM dextrose [pH 7.1]), pipetted in a small volume of HEBs buffer (in the presence or absence of BFA) onto concanavalin A-coated glass coverslips, and incubated at 37°C for 15 min. The coverslips were then quickly washed twice in HEBs buffer, and the attached monolayer of cells was subsequently fixed in 2% formaldehyde. Coated coverslips were prepared by immersing the coverslips in a freshly prepared solution of concanavalin A (Sigma, grade IV) (5 mg/ml) and water soluble carbodiimide (Calbiochem) (30 mg/ml) in 0.1 M sodium acetate (pH 5.0) for 1 hr at 25°C. The coverslips were then rinsed three times in HEBs buffer and stored in this buffer (up to 2 weeks) at 4°C before use.

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## Note Added in Proof

Fujiwara et al. have recently presented morphological evidence for the block in transport of secretory proteins out of the ER and disassembly of the Golgi complex in the presence of brefeldin A (Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. [1988]. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* **263**, 18545–18552).