The Brefeldin A-induced Retrograde Transport from the Golgi Apparatus to the Endoplasmic Reticulum Depends on Calcium Sequestered to Intracellular Stores* 

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Ribophorin I is a type I transmembrane glycoprotein specific to the rough endoplasmic reticulum. We have previously shown that, when expressed in transfected HeLa cells, a carboxyl-terminally truncated form of ribophorin I that contains most of the luminal domain (RI332) is, like the native protein, retained in the endoplasmic reticulum (ER). Brefeldin A (BFA) treatment of these HeLa cells leads to O-glycosylation of RI332 by glycosyltransferases that are redistributed from the Golgi apparatus to the ER (Ivessa, N. E., De Lemos-Chiarandini, C., Tsao, Y.-S., Takatsuki, A., Adesnik, M., Sabatini, D. D., and Kreibich, G. (1992) J. Cell Biol. 117, 949–958). Using the state of glycosylation of RI332 as a measure for the BFA-induced backflow of enzymes of the Golgi apparatus to the ER, we now demonstrate that the retrograde transport is inhibited when cells are treated with various agents that affect intracellular Ca2+ concentrations, such as the dipeptide benzyl-oxycarbonyl (Cbz)-Gly-Phe-amide, the Ca2+-ionophore A23187, and thapsigargin, an inhibitor of the Ca2+-transporting ATPase of the ER. These treatments prevent the BFA-induced O-glycosylation of RI332. Immunofluorescence localization of the Golgi markers, MG-160 and galactosyltransferase, shows that when BFA is applied in the presence of Ca2+ modulating agents, the markers remain confined to the Golgi apparatus and are not redistributed to the ER, as is the case when BFA alone is used. Cbz-Gly-Phe-amide does not, however, interfere with the BFA-induced release of β-COP from the Golgi apparatus. We conclude that the maintenance of a Ca2+ gradient between the cytoplasm and the lumen of the ER and the Golgi apparatus is required for the BFA-induced retrograde transport from the Golgi apparatus to the ER to occur.

In eukaryotic cells a series of cytoplasmic organelles, which extend from the endoplasmic reticulum (ER) to the plasma membrane and includes the Golgi apparatus, forms an endomembrane system through which flow of material can take place in both the anterograde and retrograde directions. Anterograde flow is necessary for the distribution of newly synthesized proteins throughout the cell and takes place by means of vesicles that bud from a donor organelle and fuse with an acceptor membrane (for review see Sabatini and Adesnik (1994) and Rothman (1994)). Retrograde flow, on the other hand, must be invoked as a mechanism to maintain the steady state composition of the organelles and, in particular, to return to their site of origin constitutive components (i.e. nonpassengers) of the vesicles that effect anterograde flow (for review see Lippincott-Schwartz (1993) and Pelham (1989)).

Forward transport between the ER and the Golgi apparatus is blocked by the drug brefeldin A (BFA) (for review see Klausner et al. (1992)). This drug acts by preventing the activation of the small GTP-binding protein ARF, which is required for the assembly of a protein coat on the donor membrane and the ensuing vesicle formation (Donaldson et al., 1992; Helms and Rothman, 1992). Under these circumstances, when anterograde transport is suppressed, retrograde transport from the Golgi to the ER becomes apparent or is, possibly, greatly induced. This leads to the redistribution of many resident Golgi constituents to the ER and to the nearly complete resorption of the Golgi apparatus into the ER (Klausner et al., 1992; De Lemos-Chiarandini et al., 1992). A striking manifestation of the redistribution of Golgi components to the ER is the modification by Golgi enzymes of ER resident proteins that are not normally accessible to them. These include the conversion of N-linked oligosaccharides to endo-H resistant forms and the addition of O-linked sugars to proteins susceptible to this modification (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989; Ivessa et al., 1992).

The retrograde transport that takes place in the presence of BFA is thought to be mediated by tubular processes that in drug-treated cells are seen to emerge from Golgi cisternae, rather than by discrete vesicular carriers analogous to those that mediate anterograde transport (Lippincott-Schwartz, 1993). The biochemical requirements for anterograde transport have been studied in considerable detail using semi-intact cells (Pind et al., 1994) in which it is possible to monitor the modifications of oligosaccharides that accompany the forward flow of glycoproteins. On the other hand, very little is known of the biochemical requirements for Golgi to ER retrograde transport.

In previous work (Ivessa et al., 1992) we have observed that the backflow of Golgi enzymes into the ER induced by BFA can be easily measured by following the addition of O-linked sugars
to a truncated non-membrane-anchored form (RI332) of the resident ER transmembrane protein ribophorin I. As is the case with the native protein, the truncated polypeptide is retained in the ER and does not contain O-linked oligosaccharides. In contrast to ribophorin I, which appears to be only a poor substrate for the Golgi glycosyltransferases, RI332 is efficiently modified by O-glycosylation when cells that express it are treated with BFA. This may be due to the fact that the native protein is incorporated into a macromolecular complex that renders it inaccessible to the relocated Golgi glycosyltransferases. In this paper we have monitored the O-glycosylation of RI332 to assess the effect of Ca\(^{2+}\) perturbing agents on the backflow of Golgi enzymes induced by BFA. Applying this biochemical criterion as well as results from immunofluorescence studies using Golgi markers, it was concluded that benzoyloxycarbonyl (Cbz)-Gly-Phe-amide, C23187, and thapsigargin, three agents that perturb Ca\(^{2+}\) homeostasis in cells, suppress the BFA-induced fusion of the Golgi elements with the ER. The importance of the regulation of cytosolic Ca\(^{2+}\) concentrations is supported by recent findings showing that the Ca\(^{2+}\)-binding protein calmodulin plays a role in this process (de FIGUEREDO and Brown, 1995). Our results indicate that sequestration of Ca\(^{2+}\) to intracellular stores is required for retrograde transport between the ER and the Golgi apparatus.

**EXPERIMENTAL PROCEDURES**

**Materials**—BFA was obtained from Epicentre Technologies (Madison, WI) and kept as a stock solution in methanol (5 mg/ml). The dideoxynucleosides (RI332) and the parental ribophorin I were purified from human erythroleukemic cells (5 mg/ml) and were dissolved in new, N,N-dimethylformamide (400 mM); stocks were used within 2 weeks. EGTA and the Ca\(^{2+}\) ionophore A23187 (hemimagnesium salt; stock solution: 1 mM in ethanol) were from Sigma. All stock solutions were kept at room temperature. 35S-Labeled methionine as EXPRE35S35S[35S]Protein Labeling Mix to a truncated non-membrane-anchored form (RI332) of the Golgi complex was from Bachem Bioscience Inc. (Philadelphia, PA) and was dissolved in son, WI) and kept as a stock solution in methanol (5 mg/ml). The concentration of 0.5 mM. All stock solutions were kept at 20°C for 3.5 min or fixed in 2% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature. The methanethiosulfonate reagent was rinsed in PBS and incubated with 3% nonfat dry milk (Carnation, Los Angeles, CA) in PBS (buffer A) for 15 min to block nonspecific protein binding sites. The cells fixed with paraformaldehyde were washed twice in PBS, and free aldehyde groups were quenched with 50 mM ammonium chloride in PBS for 15 min and permeabilized with 0.2% saponin in PBS containing 3% BSA (buffer B) for 15 min at room temperature. Coverslips were incubated with rabbit anti-MG-160 antibody diluted 1:125, with rabbit anti-galactosyltransferase antibody diluted 1:75 or with the ascosates of the monoclonal anti-b-COP antibody diluted 1:35 in the respective buffer, A or B, for 1 hr at 37°C. Cells used as controls were incubated with nonimmune serum. After washing three times for 10 min the coverslips were incubated with affinity-purified FITC-conjugated goat anti-rabbit F(ab')\(^{2}\)-IgG and affinity-purified FITC-conjugated sheep anti-mouse F(ab')\(^{2}\)-IgG from Organon Teknika-Cappel Research Products (Durham, NC).

**Cell Culture, Immunoprecipitation, and Data Analysis**—The permanent HeLa cell transformant (HeLa-Ri332) that expresses a truncated form of ribophorin I, comprising the 332 NH\(_{2}\)-terminal amino acids of the luminal domain of this rough ER-specific type I transmembrane glycoprotein, has been described elsewhere (Tsao et al., 1992). The methodology for cell culture, radioactive labeling, immunoprecipitation, endoglycosidase H treatment, and SDS-PAGE was reported previously (Tsao et al., 1992). When pharmacological agents were employed, control cultures received the equivalent amounts of the appropriate solvents. SDS-polyacrylamide 6–11% gradient gels were used to resolve the bands corresponding to the native RI332 and its O-glycosylated form observed after BFA treatment. The intensity of radioactivity in each band was determined by scanning densitometry of fluorograms or autoradiograms of gels using the GS370 computer program (Hoefer Scientific Instruments, San Francisco, CA) or by Phosphor Imager analysis and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Indirect Immunofluorescence**—HeLa-Ri332 cells or normal rat kidney cells, plated on coverslips at a density of 5 × 10\(^{4}\) cells/cm\(^{2}\) were left untreated or were incubated either with media containing BFA (5 μg/ml), Cbz-Gly-Phe-amide (3 mM), Cbz-Gly-Gly-amide (3 mM), or thapsigargin (0.5 μM) or with media containing a combination of BFA with one of these drugs for various time periods. The cells were either treated with 100% methanol at −20°C for 3.5 min or fixed in 2% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature. The methanethiosulfonate reagents were rinsed in PBS and incubated with 3% nonfat dry milk (Carnation, Los Angeles, CA) in PBS (buffer A) for 15 min to block nonspecific protein binding sites. The cells fixed with paraformaldehyde were washed twice in PBS, and free aldehyde groups were quenched with 50 mM ammonium chloride in PBS for 15 min and permeabilized with 0.2% saponin in PBS containing 3% BSA (buffer B) for 15 min at room temperature. Coverslips were incubated with rabbit anti-MG-160 antibody diluted 1:125, with rabbit anti-galactosyltransferase antibody diluted 1:75 or with the ascosates of the monoclonal anti-b-COP antibody diluted 1:35 in the respective buffer, A or B, for 1 hr at 37°C. Cells used as controls were incubated with nonimmune serum. After washing three times for 10 min the coverslips were incubated with affinity-purified FITC-conjugated goat anti-rabbit F(ab')\(^{2}\)-IgG diluted 1:125 or FITC-conjugated sheep anti-mouse F(ab')\(^{2}\)-IgG diluted 1:125 in buffer A or buffer B, washed with the appropriate buffer and then with PBS, and rinsed briefly with distilled water before mounting with Citifluor or FITC-guard. The cells were examined with a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence optics and photographed using Kodak TMAX-400 ASA film.

**RESULTS**

To examine the effect of Cbz-Gly-Phe-amide on the BFA-induced retrograde flow of Golgi enzymes to the ER, permanent HeLa cell transformants expressing RI332 were pulse-labeled with [35S]methionine and then incubated for 30 min in medium containing various concentrations of the dipeptide. BFA was then added (5 μg/ml), and the incubation continued for up to 90 min (Fig. 1). As we previously observed (Ivessa et al., 1992), in the absence of the dipeptide, addition of BFA led to the conversion of RI332 to a more slowly migrating form with a half-time of approximately 30 min (Fig. 1, lanes a–c and m). We have
shown (Ivessa et al., 1992) that this modification results from the O-glycosylation of R132 by the relocated Golgi enzymes. However, the addition of Cbz-Gly-Phe-amide led to a dose-dependent inhibition of the BFA-induced modification (Fig. 1, lanes d–l), and O-glycosylation was completely suppressed when the dipeptide was present at a concentration of 3 mM (lanes j–l). As may be expected from its metalloprotease inhibitor activity, in parallel with its suppression of the O-glycosylation, the dipeptide also halted the degradation of R132 as seen in the fuzzy bands in lane c or f in Fig. 1. In the presence of BFA, this degradation takes place with a half-life of approximately 40 min (Ivessa et al., 1992). The specificity of the inhibitory effects of Cbz-Gly-Phe-amide was demonstrated by the complete lack of effect of the dipeptide Cbz-Gly-Gly-amide, an inactive analogue of Cbz-Gly-Phe-amide (Strous et al., 1988; Gravotta et al., 1990; Brostrom et al., 1991; Pitt and Schwartz, 1991) on either the BFA-induced O-glycosylation or the degradation of R132 (Fig. 2A, compare lanes d, h, and l with e, i, and m, respectively). Fig. 2A also shows that the active dipeptide completely suppressed the effects of BFA even when added simultaneously with the drug. Since BFA is known to act very rapidly (Lippincott-Schwartz et al., 1989; De Lemos-Chiarandini et al., 1992), the dipeptide must exert its effects almost instantaneously.

To establish whether Cbz-Gly-Phe-amide suppresses O-glycosylation by inhibiting the glycosyltransferases or by preventing the BFA-induced retrograde transport of Golgi enzymes, we determined whether the dipeptide was effective in suppressing O-glycosylation when added after the relocation of the Golgi enzymes had taken place. To this effect, cells that were pre-treated with BFA for 30 min and then pulse-labeled and chased in the presence of BFA received either the active Cbz-Gly-Phe-amide dipeptide (Fig. 2B, lanes c, e, g, and i) or its inactive analogue (lanes b, d, f, and h) at the beginning of the chase period. In this case, the active dipeptide did not prevent O-glycosylation from proceeding, although it did cause a slight delay. These results established that the active dipeptide does not inhibit the glycosylation reaction. A direct demonstration that Cbz-Gly-Phe-amide blocks the relocation of Golgi enzymes to the ER was obtained by immunofluorescence microscopy (Fig. 3). In HeLa cells the Golgi apparatus is not a compact structure confined to a crescent-shaped perinuclear region of the cytoplasm, but rather it appears to be composed of many distinct, although smaller, Golgi complexes that are dispersed throughout the cytoplasm (De Lemos-Chiarandini et al., 1992). This is demonstrated by immunofluorescence (Fig. 3A) using an antibody to MG-160, a dialdoligonosaccharide of the medial cis-ternae of the Golgi apparatus (Craul et al., 1990; Gonatas et al., 1989). As in the case with other cell types (Lippincott-Schwartz et al., 1990), treatment of HeLa cells with BFA for 30 (D) or 60 (G) min leads to the redistribution of the Golgi marker to the ER, as indicated by the reticular fluorescence pattern of the cytoplasm obtained with the antibody to MG-160. This effect was completely abolished when the active dipeptide (F, I), but not its inactive analogue (C), was added simultaneously with BFA. Neither dipeptide when added alone, however, altered significantly the distribution of the marker observed in control cells (compare B, E, and H with control in A). Similar results (not shown) were obtained using an antibody directed against galactosyltransferase, a trans Golgi marker (Roth and Berger, 1982). Electron micrographs of cells treated with Cbz-Gly-Phe-amide in the presence of BFA show stacks of Golgi cisternae but no accumulation of vesicles (not shown), indicating that the budding stage, and not the fusion of the vesicles with ER membranes, is inhibited.

Present evidence suggests that BFA prevents anterograde vesicular transport by inhibiting the assembly of coatomers on Golgi membranes (for review see Lippincott-Schwartz, 1993). On the other hand, treatment of cells with BFA leads to the formation of tubular extensions that have been implicated in the backflow of Golgi components to the ER (Lippincott-Schwartz et al., 1989, 1990). It was conceivable that Cbz-Gly-Phe-amide inhibits the BFA-mediated retrograde transport from the Golgi apparatus to the ER by inhibiting the release of coatomer protein (COP) components such as β-COP from the Golgi membranes (Donaldson et al., 1990). Therefore, an immunofluorescence study was carried out using an anti-β-COP antibody (Allan and Kreis, 1986; Duden et al., 1991) on cells treated with Cbz-Gly-Phe-amide and BFA. In order to facilitate the interpretation of these experiments normal rat kidney cells were used, which, in contrast to HeLa cells (De Lemos-Chiarandini et al., 1992), have a discrete Golgi apparatus when immunolabeled with the anti-MG-160 antibody (Fig. 4A). As expected, in untreated control cells β-COP was concentrated mainly in the perinuclear region (B) corresponding to the Golgi apparatus (A). After treatment with BFA for 30 min the β-COP staining had a diffuse cytoplasmic granular appearance (D), and the Golgi marker MG-160 assumed the reticular pattern typical for ER staining (C). Treatment of the cells for 30 min with Cbz-Gly-Phe-amide alone did not release β-COP from the Golgi apparatus (F), and the Golgi apparatus stayed largely intact (E), although tubular extensions were seen (see arrowheads in panel E) similar to those observed immediately after BFA treatment (Lippincott-Schwartz et al., 1990). Immunostaining of cells treated with both the active dipeptide and BFA shows the typical Golgi pattern (G) with MG-160 but diffuse labeling for the cytoplasm when the antibody directed against β-COP was used (H). Similar staining patterns were seen when these treatments were extended to 60 min (not shown). Therefore, Cbz-Gly-Phe-amide exerts its inhibitory effect on the BFA-mediated retrograde transport even though β-COP has been released from Golgi membranes. Since intact microtubules were present, it was conceivable that the active dipeptide affects the backflow of Golgi components to the ER by inhibiting the assembly of coatomers on Golgi membranes. However, the time course of this effect indicates that β-COP is not released from Golgi membranes by the active dipeptide.
bulges are a prerequisite for the BFA-mediated retrograde transport from the Golgi apparatus to the ER (Lippincott-Schwartz et al., 1990; Klausner et al., 1992), we considered the possibility that Cbz-Gly-Phe-amide causes a breakdown of this cytoskeletal system. However, this was not seen when cells were analyzed by immunofluorescence using anti-\(\alpha\)-tubulin antibodies or by thin section electron microscopy (not shown).

Although it was initially thought (Strous et al., 1988) that the capacity of Cbz-Gly-Phe-amide to inhibit various processes, such as protein synthesis and the transport of secretory proteins through the cell, was a direct consequence of its inhibition of an as yet unidentified metalloprotease, it has been subsequently shown that the active dipeptide, but not its inactive analogue, affects \(\text{Ca}^{2+}\) homeostasis, causing the release of 70% of the total intracellular stores, and that it lowers the cytosolic \(\text{Ca}^{2+}\) by 30% (Brostrom et al., 1991; Kuznetsov et al., 1993). Moreover, several of the effects of the metalloprotease inhibitors can be reproduced by treatment with the \(\text{Ca}^{2+}\) ionophores A23187 (Brostrom and Brostrom, 1990) and ionomycin (Kuznetsov et al., 1993). We therefore considered the possibility that the effect of the active dipeptide on retrograde transport was a consequence of its effect on \(\text{Ca}^{2+}\) homeostasis. Cytosolic \(\text{Ca}^{2+}\) levels decrease when \(\text{Ca}^{2+}\) is removed from the extracellular medium, and this effect is enhanced by the addition of the chelating agent EGTA. We found, however, that this treatment had no effect on the BFA-induced redistribution of Golgi enzymes as assessed by the O-glycosylation of RI332 (data not shown). On the other hand, treatment of the cells with the \(\text{Ca}^{2+}\) ionophore A23187 in a \(\text{Ca}^{2+}\)-containing medium (1.8 mM), which leads to a rapid equilibration of \(\text{Ca}^{2+}\) concentrations across cellular membranes (including ER and plasma membrane) (Brostrom and Brostrom, 1990), completely prevented the BFA-induced O-glycosylation of RI332 (Fig. 5, lanes e–h). Again, this was due to an inhibition of the redistribution of the Golgi glycosyltransferases and not of their activity, since the ionophores had little inhibitory effect on O-glycosylation when added 30 min after BFA, a time at which the relocation of the enzymes had already taken place (Fig. 5, lanes i–l). It has been shown before that the active dipeptide lowers the cytosolic \(\text{Ca}^{2+}\) concentration, while the ionophore, under the conditions used (1.8 mM extracellular \(\text{Ca}^{2+}\)) raises it (Brostrom and Brostrom, 1990; Brostrom et al., 1991). Since both drugs caused an inhibition of the BFA-induced retrograde transport, it appears that the effects observed may be due to the depletion of intracellular \(\text{Ca}^{2+}\) stores. The notion that both the active dipeptide and the ionophore act by a common mechanism, possibly by causing a decrease in the \(\text{Ca}^{2+}\) concentration within the ER, was supported by an experiment in which both agents were added together at suboptimal concentrations, at which each of them appeared to only slow down the BFA-induced retrograde transport (Fig. 6, a–h). In this case the combined treatment almost completely prevented the relocation of the Golgi enzymes responsible for the O-glycosylation of RI332 (Fig. 6, lanes j–l). The fuzzier appearance of bands in lanes k and l may be due to residual glycosylation that was not completely suppressed by the two drugs after 70 and 90 min of treatment.

The observations just described led us to examine the effect

![FIG. 3. The BFA-induced redistribution of Golgi constituents to the ER is inhibited by Cbz-Gly-Phe-amide, but not by Cbz-Gly-
Gly-amide. HeLa-RI332 cells were incubated in the absence (A) or presence of 5 \(\mu\)g/ml BFA for 30 (D) or 60 min (G) at 37°C. Other cultures were treated with Cbz-Gly-Gly-amide (3 \(\mu\)M) for 60 min (B) or with Cbz-Gly-Phe-amide for 30 (E) or 60 min (H). HeLa-RI332 cells were also incubated simultaneously with BFA and Cbz-Gly-Gly-amide for 60 min (C) or with Cbz-Gly-Phe-amide (3 \(\mu\)M) for 30 (F) or 60 min (I). The cells were fixed, permeabilized, and labeled for immunofluorescence microscopy by incubating the samples sequentially with an antibody directed against MG-160, a marker of the medial cisternae of the Golgi apparatus, followed by FITC-conjugated goat anti-rabbit F(ab')2-IgG. Bar, 10.3 \(\mu\)m.](image-url)
of thapsigargin on the BFA-induced retrograde flow of Golgi enzymes. This sesquiterpene lactone is a selective inhibitor of the ER Ca$^{2+}$-ATPase, a Ca$^{2+}$ pump that normally maintains the high concentration of Ca$^{2+}$ in the lumen of the organelle (Thastrup, 1990; Thastrup et al., 1990). This drug, when added 30 min before BFA, almost completely suppressed the O-glycosylation of Rl322 (Fig. 7, compare lanes e-h with lanes a-d), but had no effect when added after the BFA-induced relocation of the enzymes had already occurred (not shown). The inhibitory effect of thapsigargin on the relocation of Golgi enzymes was also demonstrated by immunofluorescence using an antibody directed against galactosyltransferase. Treatment with thapsigargin for 30 min, however, retarded considerably the effect of BFA added subsequently. Thus, whereas 20 min of incubation with BFA alone was sufficient to totally redistribute the Golgi marker to the ER (C), in the thapsigargin-treated cells at this time (G) the Golgi pattern was only somewhat altered, showing frequent tubular processes (see arrowheads in G) emanating from Golgi cisternae similar to those that can be observed in HeLa cells after 5 min of treatment with BFA alone (Fig. 8B; see also Lippincott-Schwartz et al. (1990)). Even after treatment with BFA for 40 and 60 min (H and I, respectively) in the presence of thapsigargin the distribution of the Golgi markers had not been fully converted to that typical for ER localization. Thus, although thapsigargin suppressed the backflow of Golgi enzymes to the ER, it was not as effective as the active dipeptide or the Ca$^{2+}$ ionophore. This may reflect the fact that inhibition of the Ca$^{2+}$-ATPase by thapsigargin causes only a slow release of Ca$^{2+}$ from the ER lumen (Bian et al., 1991).

DISCUSSION

The existence of a recycling pathway that functions in the retrieval of membranes and luminal content from the Golgi apparatus to the ER has been extensively characterized (for review, see Pelham (1989, 1991). This retrograde pathway is greatly amplified when cells are treated with BFA (Klausner et al., 1992). Although a strict cytoplasmic Ca$^{2+}$ requirement for the anterograde transport from the ER to the Golgi apparatus has been established (Beckers and Balch (1989), Pind et al.
intracellular Ca$^{2+}$ homeostasis (Brostrom and Brostrom, 1990; Thastrup, 1990). Recently, the effects of calmodulin-specific antagonists on the BFA-induced retrograde Golgi apparatus to ER transport have been investigated (de Figuereido and Brown, 1995). These results support the notion that the regulation of intracellular Ca$^{2+}$ levels plays a major role in this mechanism.

In eukaryotic cells Ca$^{2+}$ has been shown to be an important second messenger (for review, see Brostrom and Brostrom (1990); Berridge (1993)). For example, increases in cytosolic Ca$^{2+}$ levels are involved in triggering the exocytosis of storage granules or of synaptic vesicles (for review see Rinderl (1992)) where synaptotagmin may function as a Ca$^{2+}$ sensor (Kelly, 1995). Mobilization of Ca$^{2+}$ from intracellular stores affects protein synthesis (Brostrom et al., 1991), and Ca$^{2+}$ sequestered in the lumen of the ER is thought to be involved in protein folding (for review see Sambrook (1990) and processing of the N-linked oligosaccharide chains of glycoproteins (Kuznetsov et al., 1992). Cytoplasmic Ca$^{2+}$ levels are regulated by different Ca$^{2+}$-ATPases located in the plasma membrane and the membranes of the ER, the mitochondria (for review see Carafoli and Chiesi (1992)), and the Golgi apparatus (Virk et al., 1985; see also Antebrick and Fink (1992) for a homologous yeast protein). Since mitochondria transport Ca$^{2+}$ with low affinity, they are thought to play no major role in the intracellular Ca$^{2+}$ homeostasis (Carafoli and Chiesi, 1992). Cells maintained at physiological concentrations have low levels of cytosolic Ca$^{2+}$, and it is thought that most of the cellular Ca$^{2+}$ is sequestered in the ER (for review see Brostrom and Brostrom, 1990). Treatment of cells with thapsigargin, a specific inhibitor of the Ca$^{2+}$-ATPase of the ER (Thastrup et al., 1990), results in the loss of Ca$^{2+}$ from the lumen of this organelle. Since under these conditions, the Ca$^{2+}$ transporting systems, especially the Ca$^{2+}$ pumps in the plasma membrane, are functional, Ca$^{2+}$ levels in the ER and the cytosol are expected to equilibrate at a low level.

We have also shown that Cbz-Gly-Phe-amide inhibits the BFA-mediated retrograde transport. This agent has the capacity to deplete cells of intracellular Ca$^{2+}$ (Lelkes and Pollard, 1987; Brostrom et al., 1991), and it has been suggested that this change of cellular Ca$^{2+}$ levels is related to the activity of metalloendoproteases (Couch and Strittmatter, 1983), which may be inhibited by active dipeptides, such as Cbz-Gly-Phe-amide (Lelkes and Pollard, 1987). Metalloendoproteases have also been directly implicated in the fusion of different cellular membranes (for review see Lennarz and Strittmatter (1991)). Histamine and catecholamines are released from mast cells or from adrenal chromaffin cells, respectively, by regulated exocytosis. These events, which are triggered by an increase of intracellular Ca$^{2+}$ concentrations, are blocked by metalloendopeptiase inhibitors (Mundy and Strittmatter, 1985; Lelkes and Pollard, 1987). Metalloendoproteases have also been directly implicated in the fusion of different cellular membranes (for review see Lennarz and Strittmatter (1991)). Histamine and catecholamines are released from mast cells or from adrenal chromaffin cells, respectively, by regulated exocytosis. These events, which are triggered by an increase of intracellular Ca$^{2+}$ concentrations, are blocked by metalloendopeptiase inhibitors (Mundy and Strittmatter, 1985; Lelkes and Pollard, 1987). Metalloendoproteases have also been directly implicated in the fusion of different cellular membranes (for review see Lennarz and Strittmatter (1991)). Histamine and catecholamines are released from mast cells or from adrenal chromaffin cells, respectively, by regulated exocytosis. These events, which are triggered by an increase of intracellular Ca$^{2+}$ concentrations, are blocked by metalloendopeptiase inhibitors (Mundy and Strittmatter, 1985; Lelkes and Pollard, 1987). Metalloendoproteases have also been directly implicated in the fusion of different cellular membranes (for review see Lennarz and Strittmatter (1991)). Histamine and catecholamines are released from mast cells or from adrenal chromaffin cells, respectively, by regulated exocytosis. These events, which are triggered by an increase of intracellular Ca$^{2+}$ concentrations, are blocked by metalloendopeptiase inhibitors (Mundy and Strittmatter, 1985; Lelkes and Pollard, 1987).
sigargin suppresses the BFA-induced redistribution of Golgi elements to the ER.

An entirely different class of agents that perturbs the Ca\(^{2+}\) homeostasis in cells are the Ca\(^{2+}\)-specific ionophores, such as A23187 or ionomycin. They increase the permeability of all cellular membranes, and at the higher extracellular levels of Ca\(^{2+}\), elevated cytosolic Ca\(^{2+}\) concentrations are observed. On the other hand, when EGTA, a chelator of divalent cations, is added to the medium in addition to the ionophore, cytosolic Ca\(^{2+}\) concentrations will be low, and intracellular Ca\(^{2+}\) stores will be depleted (for review see Brostrom and Brostrom, 1990). While A23187 in the presence of high extracellular Ca\(^{2+}\) levels is expected to result in high levels of Ca\(^{2+}\) in the cytoplasm and in the lumen of the endomembrane system, treatment with thapsigargin would lead to low Ca\(^{2+}\) concentrations in these compartments. We have shown here that both A23187 and thapsigargin inhibit the BFA-mediated retrograde Golgi to ER transport. It appears, therefore, that a Ca\(^{2+}\) gradient between the cytosol and the lumen of the endomembrane compartments is required for retrograde transport to occur. It has been shown that in cells pretreated with the Ca\(^{2+}\)-specific ionophore ionomycin, addition of BFA did not result in the conversion of the high mannose oligosaccharides of \(\alpha_1\)-antitrypsin into endoglycosidase H-resistant forms (Kuznetsov et al., 1993). The interpretation given by these authors was that ionomycin had interfered with the proper processing of the high mannose oligosaccharides, thus preventing further glycosylation steps to be carried out by the Golgi glycosyltransferases that had presumably been redistributed to the ER. However, these data are also compatible with an inhibition, by Ca\(^{2+}\)-specific ionophores, of the BFA-induced retrograde transport of Golgi glycosyltransferases, an interpretation favored by our results. In fact, a Golgi-specific Ca\(^{2+}\)-ATPase has been identified in yeast, and deletion of the corresponding gene causes pleotropic secretory defects (Antebi and Fink, 1992), suggesting that elevated levels of Ca\(^{2+}\) in the lumen of the Golgi apparatus may affect vesicular transport. Furthermore, in Golgi fractions obtained from rat mammary glands a Ca\(^{2+}\)-ATPase activity has been characterized (Virk et al., 1985), and microprobe analysis demonstrated elevated Ca\(^{2+}\) levels in the Golgi apparatus of rat exocrine pancreas cells (Roos, 1988). Since our investigations are concerned with the retrograde transport from the Golgi apparatus to the ER, it may be speculated that the drugs used to perturb Ca\(^{2+}\) homeostasis were also affecting the Ca\(^{2+}\) levels in the lumen of the Golgi apparatus.

It has been observed that Cbz-Gly-Phe-amide, but not an inactive analog, inhibits protein transport in intact cells (Strous et al., 1988; Brostrom, et al., 1991), and endosomal transport in vitro (Pitt and Schwartz, 1991). Interference with intracellular transport may be achieved not only by inhibiting fusion events at the level of the acceptor organelle but also by interfering with budding from the donor membrane. The latter

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**FIG. 8.** Treatment of cells with thapsigargin inhibits the BFA-induced redistribution of Golgi components to the ER. HeLa-R1332 cells were incubated in the absence (A) or presence of 5 \(\mu\)g/ml BFA for 5 (B) or 20 min (C) at 37 °C. Other cultures were treated with thapsigargin (0.5 \(\mu\)M) for 50 (D), 70 min (E), or 90 min (F). HeLa-R1332 cells were also preincubated with thapsigargin for 30 min and then incubated simultaneously with BFA and thapsigargin for 20 (G), 40 (H), or 60 min (I). The cells were fixed, permeabilized, and labeled for immunofluorescence microscopy by incubating the samples sequentially with an antibody directed against galactosyltransferase, a marker of the trans cisternae of the Golgi apparatus, followed by FITC-conjugated goat anti-rabbit F(ab')\(_2\)-IgG. Arrowheads in panels B and G indicate tubular extensions of the Golgi apparatus. Bar, 19.8 \(\mu\)m.
scenario is supported by experiments where semipermeabilized Madin-Darby canine kidney cells infected with influenza virus were incubated with Cbz-Gly-Phe-amide (Gravotta et al., 1990). It was found that the trans Golgi network to plasma membrane transport was inhibited mainly at early times, suggesting that the dipeptide affects the budding stage and not the fusion event (Gravotta et al., 1990). In analogy, our results demonstrating an inhibition of the BFA-induced retrograde flow from the Golgi apparatus to the ER may be due to an inhibition of the formation of vesicles and tubules, a process that depends on the tight regulation of intracellular Ca^{2+} levels.

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