Post-Golgi Membrane Traffic: Brefeldin A Inhibits Export from Distal Golgi Compartments to the Cell Surface but Not Recycling

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Abstract. Recent studies using the fungal metabolite brefeldin A (BFA) have provided important insights into the dynamics and the organization of the ER/Golgi membrane system. Here we examined the effect of BFA on the functional integrity of the distal part of the secretory pathway, i.e., transport between trans-Golgi cisternae and the cell surface. To assay export via the constitutive pathway, we followed the movement of vesicular stomatitis virus (VSV) G glycoprotein that had been accumulated in the trans-Golgi network (TGN) by incubation of infected BHK-21 cells at 20°C. Addition of BFA rapidly and reversibly inhibited cell surface transport of G protein. The block to secretion was not due to redistribution of externalized G protein to internal pools. It was also not due to collapse of TGN to the ER, since VSV G protein blocked in treated cells resided in compartments that were distinct from the ER/Golgi system. Similar effects were found with a bulk-flow marker: BFA blocked constitutive secretion of glycosaminoglycan chains that had been synthesized and sulfated in the trans-Golgi cisternae. To examine export via the regulated secretory pathway, we assayed secretion of [35S]SO₄ labeled secretogranin II from PC12 cells, a marker that has been used to study secretory granule budding from the TGN (Tooze, S. A., U. Weiss, and W. B. Huttner. 1990. Nature [Lond.]. 347:207-208). BFA potently inhibited secretion of sulfated secretogranin II induced by K⁺ depolarization. Inhibition was at the level of granule formation, since BFA had no effect on regulated secretion from preformed granules. Taken together, the results suggest that BFA blocks export via both the constitutive and the regulated pathways. In contrast, endocytosis and recycling of VSV G protein were not blocked by BFA, consistent with previous studies that endocytosis is unaffected (Misumi, Y., Y. Misumi, K. Miki, A Takatsuki, G. Tamura, and Y. Ikehara. 1986. J. Biol. Chem. 261:11398-11403).

These and earlier results suggest that the exo/endocytic pathway of mammalian cells consist of two similar but distinct endomembrane systems: an ER/Golgi system and a post-Golgi system. BFA prevents forward transport without affecting return traffic in both systems.

Protein secretion from eukaryotic cells follows a pathway by which secretory products are transferred sequentially through a series of intracellular compartments. Transport is generally believed to be mediated by vesicular carriers (Palade, 1975; Farquhar and Palade, 1981; Pfeffer and Rothman, 1987), the best documented case being secretory granules involved in the final stage of regulated secretion. Evidence is accumulating for the involvement of vesicular carriers in other transport steps. Inhibition of transport by certain mutations or inhibitors (Schekman, 1982; Orci et al., 1989; Kaiser and Schekman, 1990) results in accumulation of vesicles that have been suggested to function as transport intermediates. In addition, cell-free systems have been used to study the formation of these vesicles in vitro (Malhotra et al., 1989; Wandler-Ness et al., 1990; Groesch et al., 1990; Tooze and Huttner, 1990; Tooze et al., 1990; Rexach and Schekman, 1991; d'Enfert et al., 1991). Several of the putative transport vesicles have recently been isolated and characterized (Lodish et al., 1987; Walworth and Novick, 1987; Holcomb et al., 1988; Paulik et al., 1988; Groesch et al., 1990; Wandler-Ness et al., 1990; Tooze and Huttner, 1990; Rexach and Schekman, 1991). In some cases, these vesicles have been shown to represent bona fide transport intermediates since they are able to complete transport when incubated in a second reaction with the appropriate acceptor membrane (Groesch et al., 1990; Rexach and Schekman, 1991).

A central question is how the vesicles are generated from individual donor compartments, and whether a common set of proteins is involved in each intercompartmental transfer step. Insight into this problem has been provided by studies using a fungal metabolite, brefeldin A (BFA). BFA prevents the exit of newly synthesized proteins from the ER.

1. Abbreviations used in this paper: BFA, brefeldin A; BiP, binding protein; GAG, glycosaminoglycan; Rh-transferrin, rhodamine-conjugated human transferrin; TGN, trans-Golgi network; VSV G, G glycoprotein of vesicular stomatitis virus.
Materials and Methods

Materials

BFA was obtained from Epicentre Technologies (Madison, WI) and stored as a 10-mg/ml stock solution in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. Human transferrin, BFA was obtained from Epicentre Technologies (Madison, WI) and stored in methanol at -20°C. 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Human transferrin, BFA was obtained from Epicent
tion buffer. Cells were then homogenized by repeated passage through a 23-gauge needle and unbroken cells and nuclei were removed by centrifugation for 10 min at 1,000 g. 400 #i of each postnuclear supernatant was loaded onto a step gradient containing 1 ml each of 50, 30, 27.5, 25, 22.5, 20, 17.5, 15, 12.5, and 10% sucrose, all in D2O buffered with 10 mM Hepes, pH 7.4 (Ludish et al., 1986) to localize total VSV G. In separate experiments, VSV G was metabolically labeled as described above, and the localization of [35S]-VSV G was determined on the gradients under each condition. Similar results were observed in these experiments.

**Immunofluorescence and Rhodamine-Transferin Labeling**

BHK-21 cells were plated at 5 x 10^6 cells/well on glass coverslips and grown for 12-16 h before use. Infection with VSV ts045, accumulation of VSV G in either the ER at 40°C or TGN at 19.5°C, and transport to the cell surface at 32°C, were performed as described above. For double labeling with rhodamine-conjugated human transferrin, the cells were incubated with Rh-transferrin during the accumulation of VSV G. For immunofluorescence with mAbs, the cells were fixed for 10 min at 19.5°C, the media was aspirated from each coverslip and replaced with 2 ml of serum-free buffer growth medium before fixation at 19.5°C. After fixation for an additional 15 min at 19.5°C, the cells were washed twice with 2 ml of serum-free buffer growth medium containing 10 mg/ml of Rh-transferrin and the incubation continued for an additional hour at 19.5°C. At the end of the incubation, the cells were cooled by shifting to an ice/water bath. Washed twice with 10 ml of ice-cold PBS, twice with 10 ml of ice-cold 10 mM acetic acid (pH 4.5) to remove Rh-transferrin bound to the cell surface, and then three times with ice-cold PBS. The cells were fixed for 15 min at room temperature with 2 ml of 3% paraformaldehyde, 0.02% glutaraldehyde in PBS. Cells were permeabilized after fixation by incubating for 10 min in 20°C methanol. After fixation and permeabilization the coverslips were washed three times in PBS, incubated for 10 min in 0.1% NaBH4 in PBS at room temperature, and then washed three times in PBS. The coverslips were then incubated for 30 min at room temperature in a 1:100 dilution of a mouse mAb that recognizes the lumenal domain of VSV G (Lefrancois and Lyles, 1982). After washing three times in PBS, the cells were incubated for 30 min at room temperature in a 1:25 dilution of fluorescein-conjugated goat anti-mouse antibody, washed with PBS, and mounted. Primary and secondary antibodies were diluted in PBS containing 0.2% gelatin.

**Internalization of VSV G from the Cell Surface**

BHK-21 cells were plated at 5 x 10^6 cells/well on glass coverslips and grown 12-16 h before use. The cells were infected for 45 min with 10 PFU/cell of VSV ts045 as described above, and then incubated for 2.5 h at 39.5°C to accumulate VSV G in the ER. All subsequent incubations contained 100 µg/ml cycloheximide. The cells were then incubated at 19.5°C for 2 h to mimic conditions used in BFA experiments, or directly shifted to 32°C for 1 h to accumulate VSV G on the cell surface. The cells were then incubated in fresh medium containing either 0 or 5 µg/ml BFA for 1 h at 32°C, and then fixed, permeabilized, and processed for indirect immunofluorescence of VSV G as described above.

**Antibody Cross-linking and Internalization of VSV G at the Cell Surface**

BHK-21 cells plated on glass coverslips were infected with VSV ts045, and VSV G protein was accumulated on the cell surface as described above. The cells were then shifted to an ice/water bath and each coverslip was incubated in 100 µg/ml buffer growth medium containing a 1:100 dilution of a mouse monoclonal ascites fluid specific for the extracellular domain of VSV G (Lefrancois and Lyles, 1982). Excess antibody was then removed by washing three times in ice-cold buffer growth medium. Cells were then incubated for an additional hour at 32°C in buffer growth medium containing either 0 or 5 µg/ml BFA. One set of cells was kept at 4°C during this incubation as control, and a second set of cells was incubated for 1 h at 32°C in buffer growth medium containing 20 mM 2-deoxyglucose and 10 mM sodium azide to deplete intracellular ATP. The cells were then fixed as described above, and permeabilized for 10 min in 20°C methanol. The cells were then incubated for 30 min at room temperature with a 1:25 dilution of fluorescein-conjugated goat anti-mouse antibody, washed with PBS, and observed using a fluorescence microscope.

**Metabolic Labeling and Analysis of Regulated Secretion of Secretogranin II**

PC12 cells grown in 12-well plates were starved for sulfate by incubating for 30 min in a sulfate-free buffer (buffer A: 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na2HPO4, 20 mM Hepes, pH 7.2, 10 mM MgCl2, 2 mM CaCl2, and 1 g/liter glucose). The cells were then pulse-labeled in 250 µl of buffer A containing 1 µCi/ml [35S]SO4 for 5 min at 37°C to label secretogranins and sulfated proteoglycans. Cells were chased for the indicated time in a low potassium transport buffer (buffer B: 127 mM NaCl, 5 mM KCl, 0.33 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, 20 mM Hepes, pH 7.2, 2.2 mM CaCl2, 56 mM glucose) to allow the accumulation of regulated vesicles (Rosa et al., 1985). In some experiments, BFA was added to the chase medium at a concentration of 5 µg/ml. To induce release from regulated granules, cells were depolarized with a high potassium buffer (buffer C) with composition identical to buffer B, except that the concentration of KCl was raised to 55 mM and the concentration of NaCl decreased to 77 mM. Media samples were collected, precipitated with 10% TCA, and then centrifuged for 30 min at 3,000 rpm in a GPR centrifuge (Beckman Instruments, Inc., Fullerton, CA.). The pellet was resuspended in 1 ml of 20°C acetone, and centrifuged for 5 min; the supernatant was aspirated and allowed to dry. The pellet was then boiled for 5 min in Laemmli sample buffer (Laemmli, 1970). Cells were extracted with 100 µl of NDE.T (1% NP-40, 0.4% [wt/vol] deoxycholate, 66 mM EDTA, and 10 mM Tris, pH 7.4) to each well. The extracted cells were transferred to a microcentrifuge tube and centrifuged for 5 min in a microcentrifuge to pellet insoluble debris. One tenth of the extract was added to an equal volume of 2x Laemmli sample buffer and boiled for 5 min. The media and extract samples were separated on 12.5% SDS-polyacrylamide gels, dried, and exposed to a phosphorimagery screen.

**[35S]Sulfate Labeling and Analysis of Constitutive Secretion of Glycosaminoglycan Chains**

BHK cells grown on 12-well plates (5 x 10^6 cells/well) were incubated in buffer A containing 500 µM 4-methylumbelliferyl-β-D-xyloside for 30 min at 37°C. The cells were pulse-labeled for 2 min at 37°C with buffer A containing 200 µCi/ml [35S]SO4 in the presence of xyllose, and chased in buffer A containing 5 mM Na2SO4 and 5 µg/ml BFA. At times indicated, the media samples were collected, and the cells were either extracted with Laemmli sample buffer for analysis by PAGE (Brion et al., 1992), or extracted with Triton X-100 for filtration assay using cetylpyridinium chloride (Miller and Moore, 1991). For analysis by PAGE, 10 µg chondroitin sulfate was added to each media sample as carrier, and the samples were precipitated with acetone before loading onto gels as described (Brion et al., 1992).

**Results**

**Constitutive Transport of VSV G Protein from the 20°C Compartment to the Cell Surface Is Blocked by BFA**

Since BFA blocks transport from the ER to Golgi complex, to test its effects on transport from the TGN to the cell surface we needed to examine cargo molecules that already reside within the TGN before BFA treatment. We therefore examined the fate of VSV G protein that had been accumulated in the TGN by incubating VSV-infected cells at 20°C. We used BHK-21 cells for these studies, because Griffiths et al. (1985) have performed detailed immunoelectron microscopic studies showing that in these cells VSV G accumulates in the TGN when incubated at 20°C. BHK-21 cells were infected with the temperature-sensitive ts045 mutant of VSV and incubated at 40°C for 3.5 h to accumulate VSV G in the ER (Bergmann, 1989). Subsequent chases were carried out...
Figure 1. Immunofluorescence localization of VSV G proteins and β-COP in BHK-21 cells that had been infected with VSV ts045 and blocked at 20°C. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, and incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, and again for 2 h in the presence of cycloheximide at 19.5°C to accumulate VSV G in the TGN. The localization of VSV G proteins was compared with the Golgi marker, β-COP, by double indirect immunofluorescence. The cells were fixed, permeabilized with methanol, and reacted with affinity-purified rabbit antibodies against β-COP, and a mAb against the cytoplasmic tail of VSV G protein. The locations of these proteins were then visualized using appropriate fluorescent secondary antibodies. (a-f) Two sets of cells double stained for VSV G protein (b and e) and β-COP (c and f). VSV G was visualized with fluoresceinated goat anti-mouse, and β-COP with rhodamine goat anti-rabbit antibodies. β-COP was visualized with fluoresceinated goat anti-rabbit antibody. Bar, 20 μm.

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Figure 2. BFA inhibits transport of VSV G from the 20°C compartment to the cell surface. BHK-21 cells were infected and incubated at 20°C to allow accumulation of VSV G proteins in the TGN as in Fig. 1. Cells were then incubated for 15 (a–d), 60 (e–h), or 120 min (i–l) either in the absence (a, b, e, f, i, and j) or presence (c, d, g, h, k, and l) of 5 μg/ml BFA plus cycloheximide. The cells were then fixed and VSV G that had been transported to the cell surface was detected by indirect immunofluorescence using an antilumenal antibody without permeabilization. (a, d, e, h, i, and l) Phase; (b, c, f, g, j, and k) fluorescence. Bar, 60 μm.

bright staining of the cell surface (compare Fig. 4 A, b to Fig. 2, c, g, and k). When cells were permeabilized before antibody incubations, little or no internal staining was observed in either control or BFA-treated cells (not shown). To rule out this possibility that the G protein behaves differently in cells that had been incubated at 20°C (since experiments in Fig. 2 and Fig. 3 had this step), we performed the same experiment on cells that had been incubated at 20°C for 2 h. Essentially the same results were obtained: cells that had been treated with BFA (Fig. 4 B, e and f) show the same bright cell surface staining as before the treatment (Fig. 4 B, a and b) or mock-treated cells (Fig. 4 B, c and d). Staining of permeabilized cells also showed no detectable differences between control and BFA-treated cells (not shown). These observations rule out the possibility that BFA shifts the steady-state distribution of VSV G from the surface to intracellular pools. Taken together, these results indicate that constitutive transport from the TGN to the cell surface is significantly inhibited by BFA.

VSV G Protein Blocked at 20°C Resides in a Compartment Distinct from the ER and Early Endosomes after BFA Treatment

If transport between the TGN and the cell surface is blocked by BFA, what is the fate of VSV G that had been accumulated in the TGN? We examined the intracellular localization of
VSV G protein that had been accumulated in the TGN but prevented from reaching the cell surface by BFA. Cells were infected with VSV ts045, chased in the presence of cycloheximide at 20°C for 2 h, and then shifted to 32°C in the presence or absence of BFA. Indirect immunofluorescence of fixed, permeabilized cells show that in BFA-treated cells, VSV G protein was found in vesicular structures with altered morphology from untreated cells (compare Fig. 5 A, b–d with a), often concentrated in perinuclear region. Some of these structures may colocalize with the microtubule organizing center (also see Fig. 8), similar to the structures seen by Reaves and Banting (1992) using a TGN marker, TGN-38, in BFA-treated normal rat kidney cells. Untreated, control cells rapidly lost internal staining as most of the G protein was chased to the cell surface (not shown). Note that in BFA-treated cells, little or no staining of the ER was observed. This pattern is in marked contrast to experiments in which BFA was added during the 20°C incubation; in this case diffuse reticular staining characteristic of the ER was observed throughout the cytoplasm, in addition to bright staining of the nuclear envelope (Fig. 5 B, b). Thus, VSV G resides in a compartment distinct from the ER after BFA treatment. This point was confirmed by subcellular fractionation. VSV-infected BHK-21 cells were chased at 20°C for
2 h in the presence of cycloheximide, and then shifted to 32°C for 1 h in the presence or absence of BFA. The cells were then homogenized, and the postnuclear supernatant was separated on sucrose/D₂O gradients (Lodish et al., 1987). Fig. 6 shows that VSV G in BFA-treated cells sedimented at a density that was distinct from the ER membranes. The ER membranes, as marked by either VSV G blocked at 40°C (Fig. 6 a) or antibodies to the luminal ER protein BiP (Fig. 6, d and f) (Bole et al., 1986), were predominantly found in fractions 2, and 3, whereas VSV G protein in treated cells sedimented in fractions 4–6 (Fig. 6 e). These results indicate that the block to export from the TGN to the cell surface is not simply caused by the collapse of the TGN back to the ER. Instead, the TGN represents a separate
Figure 5. VSV G accumulated in the 20°C compartment does not redistribute to the ER in the presence of BFA. (A) BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, and then for 2 h in the presence of cycloheximide at 19.5°C to accumulate VSV G in the TGN. Cells were then incubated for 0 (a), 15 (b), 60 (c), or 120 min (d) at 32°C in the presence of 5 μg/ml BFA plus cycloheximide. The cells were then fixed and permeabilized, and internal VSV G was detected by indirect immunofluorescence. (B) BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, and then incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER. Cells were then incubated for 2 h at 19.5°C in either the absence (a) or presence (b) of 5 μg/ml BFA plus cycloheximide. The cells were then fixed and permeabilized, and internal VSV G was detected by indirect immunofluorescence. Bars, 20 μm.

entity distinct from the ER/Golgi membrane system, as has been previously suggested (Chege and Pfeffer, 1990). Recently, BFA has been found to affect the endosomal system, causing early endosomes to redistribute to a perinuclear location near the microtubule organizing center (Lippincott-Schwartz et al., 1991). Since the VSV G-containing structures in BFA-treated cells are also often localized in this region of the cell (see Fig. 5 A), we examined the distribution of endosomal structures labeled with Rh-transferrin in BFA-treated cells and compared it with those containing VSV G.
Figure 6. Subcellular fractionation of VSV G and BiP after treatment with BFA. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER (a), and again for 2 h at 19.5°C in the presence of cycloheximide to accumulate VSV G in the TGN (b). The cells were then chased for 1 h at 32°C in the absence (c and d) or presence (e and f) of 5 μg/ml BFA plus cycloheximide. For each condition, cells were homogenized and postnuclear supernatants were fractionated on sucrose/D2O gradients. The proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for either VSV G (a-c and e) or BiP (d and f). (a) VSV G after 3.5 h at 39.5°C; (b) VSV G after 2 h at 19.5°C; (c) VSV G after 1 h at 32°C in the absence of BFA; (d) BiP after 1 h at 32°C in the absence of BFA; (e) VSV G after 1 h at 32°C in the presence of BFA; and (f) BiP after 1 h at 32°C in the presence of BFA.

We first examined the effect of BFA treatment on the intracellular distribution of Rh-transferrin. BHK-21 cells were labeled with Rh-transferrin for 1 h at 37°C and then incubated for varying times in the presence of 5 μg/ml BFA before fixation and examination using a fluorescence microscope. Fig. 7 shows that BFA causes a rapid redistribution of Rh-transferrin-labeled structures into tubular structures. By 2 h the Rh-transferrin was found in a dense meshwork of tubular structures, often in the perinuclear region of the cell. Double-labeling experiments in VSV-infected cells showed that these structures, although localized to similar regions within the cell, were distinct from those containing VSV G (Fig. 8). Thus, VSV G blocked within BFA-treated cells resides within a compartment that is largely distinct from early endosomes labeled by Rh-transferrin. At this point, we cannot rule out the possibility that the structures containing VSV G protein are in communication with endosomes, as has been shown for TGN-38 and mannos-6-phosphate receptor (Lippincott-Schwartz et al., 1991; Wood et al., 1991). The effects of BFA on these post-Golgi endomembrane systems is completely reversible; upon removal of BFA, VSV G was rapidly transported to the cell surface and the endosomal compartment labeled by Rh-transferrin returned to its normal morphology (data not shown).

Endocytosis of VSV G and Rh-Transferrin Continues in the Presence of BFA

The above data suggest that export from the TGN to the cell surface via the constitutive pathway is blocked by BFA. VSV G is known to be internalized from the cell surface into early endosomes, where it either recycles to the cell surface or is directed to lysosomes (Gottlieb et al., 1986; Gruenberg and Howell, 1987). When VSV G on the cell surface is cross-linked by antibodies, recycling to the cell surface is abolished and internalized VSV G is targeted to the lysosomal pathway, resulting in a rapid loss of VSV G from the cell surface (Gruenberg and Howell, 1987). To test whether endocytosis continues in the presence of BFA, BHK-21 cells were infected with VSV ts045; and VSV G was accumulated in the ER by incubation at 39.5°C for 2.5 h and then chased in the presence of cycloheximide at 32°C for 1 h to allow transport to the cell surface. An antibody that recognizes the extracellular domain of VSV G was added to the cells at 4°C for 1 h. Excess unbound antibody was removed and the cells were warmed to 32°C for 1 h to allow endocytosis to occur. The cells were then fixed and permeabilized, and internalized antibody was visualized with a fluorescent secondary antibody (Fig. 9). In both control and BFA-treated cells, the antibodies were found in large, extremely bright, vesicular structures and a concomitant decrease in cell surface staining was observed. No difference was detected between control and BFA-treated cells. These structures were not detected when the permeabilization step was omitted (not shown), and their formation required energy, since incubation at 32°C in the presence of 2-deoxyglucose and sodium azide prevented internalization (Fig. 9, a and b). The internal VSV G–containing structures bear a striking resemblance to those observed...
Figure 7. BFA causes redistribution of early endosomes into tubular structures. BHK-21 cells were incubated for 1 h at 37°C in serum-free medium containing 10 μg/ml Rh-transferrin; BFA was added to a final concentration of 5 μg/ml; and the incubation was continued for 0 (a), 15 (b), 60 (c), or 120 min (d) at 37°C. The cells were then washed to remove external Rh-transferrin, fixed, and observed by fluorescence microscopy. Bar, 20 μm.
when cells expressing VSV G were incubated with chloroquine to prevent recycling of endocytosed VSV G to the cell surface (Gottlieb et al., 1986). We conclude that VSV G is still internalized in the presence of BFA. This result was further confirmed by using Rh-transferrin. Incubation of cells pretreated in BFA with Rh-transferrin resulted in uptake of the marker into reticular structure similar to those shown in Fig. 7, indicating that endocytosis into early endosomes was not blocked (not shown). These results are consistent with those obtained by Misumi et al. (1986), who showed that endocytosis and degradation of asialofetuin was not affected.

Two lines of evidence suggest that recycling from the early endosomal compartment to the cell surface is not blocked by BFA. First, such a block would lead to the loss of VSV G from the cell surface, as has been reported when this step is blocked by chloroquine (Gottlieb et al., 1986); this did not occur in the presence of BFA (see Fig. 4). Second, endocytosed Rh-transferrin was rapidly lost from the cells upon removal of extracellular Rh-transferrin, even in the presence of BFA (not shown), suggesting that recycling was not impaired by BFA. Thus, trafficking between the cell surface and endosomes remains functional in the presence of BFA.

Constitutive Secretion of Sulfated Glycosaminoglycan Chains Is Blocked by BFA

In the above experiments, we examined transport of a membrane-bound cargo protein. To test the effect of BFA on soluble molecules, we determined the effects of BFA on secretion of sulfated glycosaminoglycan (GAG) chains. We
Figure 9. BFA does not block endocytosis of cell surface VSV G protein cross-linked with antibodies. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 2.5 h at 39.5°C to accumulate VSV G in the ER, and then incubated for 1 h in the presence of cycloheximide at 32°C to accumulate VSV G on the plasma membrane. The cells were then incubated at 4°C for 1 h with an mAb that recognizes the extracellular domain of VSV G. The cells were washed to remove unbound antibody, warmed to 32°C for 1 h in the presence of cycloheximide, and fixed; and VSV G was localized using a fluorescein-conjugated secondary antibody after permeabilization. (a and b) Cells incubated at 32°C in the presence of 2-deoxyglucose and sodium azide; (c and d) control cells incubated at 32°C; (e and f) cells incubated at 32°C in the presence of 5 μg/ml BFA. Bar, 20 μm.

have shown previously that GAG chains serve as a convenient bulk-flow marker for the constitutive pathway (Miller and Moore, 1991). GAG chains are sulfated in the trans Golgi thus sulfated GAG chains can be used to monitor transport between distal Golgi compartments and the cell surface. When BFA was added to the cells before pulse-labeling with [35S]SO4, no labeled GAG chains could be detected in the cells. This is consistent with the recent findings that BFA uncouples initiation of GAG chain synthesis from elongation and sulfation, presumably because the former process occurs early in a Golgi compartment that collapses to the ER, whereas the latter processes occur later in Golgi cisternae that stay distinct from the ER (Spiro et al., 1991). To examine whether fully elongated, sulfated GAG chains synthesized in the absence of BFA can be secreted upon BFA treatment, we first incubated the cells with xyloside and pulse-labeled them with [35S]SO4 for 2 min to allow synthesis of fully elongated, sulfated GAG chains. The cells were then chased in medium containing 5 μg/ml BFA. As shown in Fig. 10 A, BFA potently blocked secretion of GAG chains. The sulfated chains remaining in the cells had the same mobility on SDS-PAGE as in control cells; no degradation was detected, as would be expected if they were diverted to other organelles such as lysosomes. The kinetics of GAG chain secretion was analyzed by a precipitation filtration assay (Miller and Moore, 1991). The result (Fig. 10 B) shows that BFA inhibited >96%
BFA blocks secretion of [35S]SO4 GAG chains via the constitutive pathway. (A) BHK-21 cells grown in 12-well plates were pre-incubated with 0.5 mM xyloside for 30 min at 37°C, and pulse-labeled with [35S]SO4 for 2 min. The cells were then chased in medium containing 5 µg/ml BFA for 0, 15, or 30 min. Media and extract samples were analyzed by 18% PAGE (see Materials and Methods). Media and extract samples from (lanes 1-6) control cells, or (lanes 7-12) BFA-treated cells. (B) Secretion quantitated by cetylpyridinium chloride filtration assay. Cells were pulse-labeled as in A, and chased for 0, 10, 20, 30, 40, 50, and 60 min in medium containing 5 µg/ml BFA. Cells were extracted, and the amount of labeled GAG chains in media and extract samples were assayed by cetylpyridinium chloride precipitation and filtration. The fraction of total GAG chains secreted is plotted as a function of time. The total amount of labeled GAG chains recovered is 150,000 ± 12,000 cpm (n = 6) in control cells, and 140,000 ± 4,000 cpm (n = 6) in BFA-treated cells.

Secretion of Sulfated Secretogranin II Is Blocked by BFA

We next examined the effect of BFA on transport from the TGN to the cell surface via the regulated secretory pathway. We utilized PC12 cells for these studies since this cell line is known to secrete secretogranins by the regulated secretory pathway (Rosa et al., 1985). Moreover, secretogranins are modified by tyrosine sulfation, a process believed to occur in the trans-most Golgi cisternae (Baeuerle and Huttner, 1987; Niehrs and Huttner, 1990). Thus, transport between this compartment and the cell surface can be monitored using sulfated secretogranins. PC12 cells were pulse-labeled with [35S]SO4, for 5 min, and then chased in the presence of BFA for 1 h to allow the accumulation of 35S-labeled secretogranin in regulated secretory vesicles. The cells were then depolarized by incubation for 15 min in medium containing high K+ Ca2+ to induce the regulated secretion of secretogranins. Fig. 11 A shows that in control, untreated cells, K+ depolarization stimulated secretion from a basal level of 0.6% to 6.3% (Fig. 11 A, lanes 3 and 4). If, however, after pulse-labeling the cells were chased in the presence of BFA, secretion was almost completely blocked (Fig. 11 B, lanes 3 and 4): <0.6% of total labeled secretogranin II was secreted from either K+ -stimulated or nonstimulated cells— or at least 10-fold lower than control cells. Notice that PC12 secretes a sulfated proteoglycan via the constitutive pathway (Tooze and Huttner, 1990; see Fig. 11 A, lanes 1 and 2, the smear migrating just below the start of the separation gel); in treated cells, secretion of this sulfated proteoglycan was also dramatically reduced (Fig. 11 B, lanes 1 and 2). Thus, BFA potently inhibited export from the constitutive and regulated secretory pathways. The block is most likely due to inhibition of transport out of the TGN rather than a block to fusion of regulated secretory granules (Fig. 11 C shows that if the 35S-labeled secretogranin was first chased to mature granules before the addition of BFA, the drug no longer inhibited secretion from the regulated pathway.

Discussion

Intracellular transport in eukaryotic cells is accomplished by sequential transfer between successive compartments. Each transport step involves very similar processes, i.e., generation of vesicular intermediates, targeting of these vesicles to the appropriate compartment, and docking/fusion. The similarities suggest that some components of the transport machinery may be used at multiple steps, while others must be unique to individual step to ensure the specificity of transport. Earlier results suggested that the target for BFA might only function in early, but not in late, secretory compartments. In these earlier studies, however, the effect of export from distal Golgi compartments was not examined. In this
Figure 11. Entry of $[^{35}S]$SO$_4$ secretogranin II into the regulated secretory pathway is inhibited by BFA. PC12 cells on a 12-well plates were pulsed-labeled with $[^{35}S]$SO$_4$ for 5 min and chased in unlabeled medium containing low K$^+$ for 1 h to allow packaging of labeled secretogranins into storage granules (lanes 1 and 2 in each panel). The cells were then incubated in medium containing high K$^+$ and Ca$^{2+}$ for 15 min to stimulate release from storage granules (lane 4 in each panel). The level of unstimulated release was measured by incubating one set of wells in medium containing low K$^+$ during this 15 min (lane 3 in each panel). Media (lanes 1-4) and extract samples (lanes 5 and 6) were precipitated with TCA and the total media samples and one tenth of the extract samples were separated by SDS-PAGE. The gels were dried and $^{35}$S-labeled proteins detected using a phosphorimager. (A) Control cells incubated in the absence of BFA. (B) Cells treated with 5 µg/ml BFA during both chases. (C) Cells chased for 1 h in the absence of BFA to accumulate labeled secretogranins in the storage granules, and then incubated in the presence of 5 µg/ml BFA during the 15-min stimulation period to examine its effect on release from preformed granules. (Lanes 1 and 2) Media samples collected during the first 1 h of chase. (Lanes 3 and 4) Media collected during the subsequent 15 min from nonstimulated and stimulated cells, respectively. (Lanes 5 and 6) Cell extracts from the corresponding nonstimulated and stimulated cells, respectively, extracted at the end of the chase periods.
paper, we used both soluble and transmembrane markers (sulfated secretogranins, sulfated GAG chains, and VSV G protein blocked at TGN at 20°C) to demonstrate that export from the distal Golgi compartments to the cell surface is potentially and reversibly blocked by BFA. Since export from the ER is similarly inhibited, we suggest that ER to Golgi and Golgi to cell surface transport may share some component that is either identical or homologous; BFA affects such a component(s) and thus blocks export from both compartments. The situation would be similar to the N-ethylmaleimide-sensitive factor (Block et al., 1988) or the yeast Sec18 gene product (Eakle et al., 1988; Wilson et al., 1989), which functions at multiple steps (Block et al., 1988; Beckers, 1989; Graham and Emr, 1991; Diaz et al., 1989). In contrast to these generalized factors, involvement of small molecular weight GTP-binding proteins in transport is step specific (for review see Balch, 1990). Likewise, Sec23p appears to participate in vesicle budding only in early secretory compartments of yeast cells (Kaiser and Schekman, 1990; Graham and Emr, 1991) and is localized specifically to the ER transitional elements, but not the Golgi elements, in mammalian cells (Orci et al., 1991).

Using TGN-38 and mannose-6-phosphate receptor as markers for the TGN, Lippincott-Schwartz et al. (1991) and Wood et al. (1991) showed that BFA induces mining of TGN with early endosomes, resulting in an increase in cell surface mannose-6-phosphate receptor and uptake of anti-TGN-38 antibodies. Thus, at least for these two proteins, traffic between TGN and the cell surface appears to continue in BFA. In this regard, it is surprising that transport of VSV G and sulfated GAG chains and secretogranins to the cell surface is inhibited. There are two possible explanations. First, TGN-38 and mannose-6-phosphate receptor may reside in a different compartment from VSV G at 20°C or sulfated GAG chains and secretogranins. For VSV G protein, however, this is not very likely since it has been colocalized to the same compartment as TGN-38 in NRK cells by immunoelectron microscopy (Luzio et al., 1990). Another possibility is that TGN-38 and mannose-6-phosphate receptor may have a different itinerary from VSV G protein and secreted proteins (for instance, at least mannose-6-phosphate receptor normally recycles between endosomes and TGN), and are thus affected differently by BFA. Future work is necessary to distinguish between these possibilities.

Several proteins of the transport machinery have been shown to be affected by BFA (Donaldson et al., 1990). One of these, β-COP (Waters et al., 1991; Allan and Kreis, 1986; Malhotra, 1989; Serafini et al., 1991), is a component of the coat found on nonclathrin-coated vesicles. One possibility is that this coat complex participates in multiple steps, generating forward-bound transport vesicles from ER transitional elements, Golgi cisternae, and the TGN. BFA causes redistribution of this coat complex from the membrane to the cytosol, thereby preventing forward movement of proteins from the ER to the Golgi cisternae and also from the TGN to the cell surface. In support of this view, β-COP is found to colocalize with VSV G protein when export from the ER and TGN is blocked at 15°C and 20°C, respectively (Duden et al., 1991b; Miller, S. G., and H.-P. Moore, unpublished observations). This distribution is consistent with a role in both export from the ER and the TGN. The time course of inhibition is also consistent with this hypothesis; inhibition
ward traffic stops but return traffic continues, suggesting similar structural organizations. Normally, these two systems must be in communication in the forward direction since proteins in the biosynthetic pathway traverse Golgi compartments and the TGN. BFA blocks this forward transport, severing communication between the two systems. The fact that the two systems do not become intermingled also suggests that either there is little or no backward transport between the systems, or, if it exists, the mechanism must be quite different from that operating between other Golgi cisternae. This could provide a regulatory mechanism to control communication between the "outer" and "inner" membrane systems.

In summary, much remains to be learned about the biochemical components that mediate protein traffic through the constitutive and regulated exocytic pathways as well as in the endocytic pathway. However, the data presented in this paper provide some important constraints about the components involved in the exo/endo systems. Future experiments will be important to address questions such as whether the coatamer complex functions as a budding component or as a scaffold, and whether Sec12p like protein(s) function in multiple steps and is the primary target for BFA.

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