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 $[^{35}S]SO_4$ 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; Wandinger-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; Wandinger-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).[1] 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.

(Misumi et al., 1986; Oda et al., 1987). In addition, it leads to rapid disruption of Golgi structure and microtubule-dependent retrograde transport of *cis*-, medial-, and *trans*-Golgi enzymes back to the ER (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Bosshart et al., 1991; Russ et al., 1991). These results suggest that BFA blocks forward transport between the ER and Golgi complex while allowing retrograde transport to continue. An exciting finding is that BFA causes rapid (<15 s) dissociation of a 110-kD protein from Golgi membranes (Donaldson et al., 1990). This protein, β-COP, has been shown to be a component of the nonclathrin-coated vesicles that accumulate when intercisternal Golgi transport is blocked by GTPγS (Malhotra, 1989; Serafini et al., 1991). BFA prevents formation of this type of vesicle in vitro (Orci et al., 1991), supporting the notion that β-COP participates in coat assembly. Interestingly, β-COP shares some sequence identity with β-adaptin (Duden et al., 1991*a*,*b*), a protein involved in the assembly of clathrin coats (for reviews see Pearse and Crowther, 1987; Ponnambalam et al., 1990). Like the clathrin coats, β-COP was found to be present in a multisubunit protein complex, termed "coatomer" (Waters et al., 1991). These findings are consistent with a model that β-COP, and therefore the coatomer complex, plays an essential role in the forward transport between the ER and Golgi complex, and between early Golgi compartments.

Whether β-COP and the coatomer complex also function in distal parts of the Golgi complex is not clear. Data thus far suggests that BFA does not affect the *trans*-Golgi network (TGN). For example, in contrast to the proximal Golgi cisternae, the TGN does not appear to collapse into the ER, since newly synthesized proteins blocked in the ER are not modified by the TGN-resident enzyme, sialyltransferase (Doms et al., 1989). Furthermore, mannose-6-phosphate receptors labeled at the cell surface can still return to the site of sialylation in the presence of BFA, indicating that endocytic traffic from the cell surface to the TGN is unaffected (Chege and Pfeffer, 1990). On the other hand, double immunofluorescence localization using antibodies specific for β-COP and the G glycoprotein of vesicular stomatitis virus (VSV G) demonstrated that these two proteins are colocalized when biosynthetic transport is blocked at either 15°C (a pre-Golgi compartment) or 20°C (TGN) (Duden et al., 1991*b*; Miller, S. G., and H.-P. Moore, unpublished observations). In this paper, we address the question of whether exit from the TGN can still occur in BFA-treated cells. We find that transport from *trans*-Golgi compartment to the cell surface, via both the constitutive and regulated pathways, is rapidly inhibited upon treatment with BFA. In contrast, endocytosis from the cell surface continues. Thus, BFA exerts similar effects on pre- and post-Golgi traffic, blocking forward transport but not return traffic.

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, coupled to tetramethylrhodamine, was purchased from Molecular Probes, Inc. (Eugene, OR). BHK-21 cells were obtained from the American Type Culture Collection (Bethesda, MD). PC12 cells were obtained from Dr. Stuart C. Feinstein (University of California, Santa Barbara, CA). Mouse mAb 865, specific for the extracellular domain of VSV G, was provided by Dr. L. Lefrancois (Scripps Clinic and Research Foundation, La Jolla, CA; Lefrancois and Lyles, 1982). A rat mAb specific for the Ig heavy chain binding protein (BiP) was the kind gift of Dr. D. Bole (University of Michigan, Ann Arbor, MI. Rabbit anti-β-COP was generated against a peptide corresponding to the VSALGIDFKVKTIYR sequence of β-COP (Duden et al., 1991*a*,*b*). The peptide was conjugated to keyhole limpet hemocyanin via an NH_2-terminal cysteine residue, and the antibodies were purified on a peptide affinity column. Fluorescein-conjugated secondary antibodies were purchased from Kirkegaard and Perry Labs., Inc. (Gaithersburg, MD).

Cell Culture

BHK-21 cells were maintained at 37°C in a 5% CO_2 atmosphere in MEM supplemented with Earle's balanced salt solution containing 10% tryptose phosphate broth and 10% FCS. PC12 cells were maintained in DME containing 5% enriched calf serum and 5% horse serum under 10% CO_2 atmosphere. The cells were plated onto poly D-lysine-coated (50 μg/ml) 12-well plates at a density of 5×10^5 per well and grown for 3–4 d before experiments.

Metabolic Labeling and Cell Surface Immunoprecipitation of VSV G

BHK-21 cells were plated at a density of 2×10^6 cells per 10-cm dish and grown until near confluency (~48 h). The cells were washed three times in serum-free growth medium buffered with 25 mM Hepes, pH 7.4, and incubated for 45 min at 32°C in 2 ml of the same medium containing 10–15 PFU/cell of a temperature-sensitive mutant of VSV, ts045. The medium was then removed, and 10 ml of growth medium containing 10% FCS and 25 mM Hepes, pH 7.4, prewarmed to 39.5°C, was added. The cells were then incubated for 3.5 h at 39.5°C to allow the accumulation of VSV G in the ER. The cells were washed twice with 5 ml of Ca^{2+}/Mg^{2+}-free PBS (prewarmed to 40°C) and incubated for 5 min at 40°C in 1 ml of Ca^{2+}/Mg^{2+}-free PBS supplemented with 5 mM EDTA to detach the cells. Suspended cells were transferred to a 1.5-ml polypropylene centrifuge tube, pelleted by spinning briefly in a microcentrifuge, and resuspended in 1 ml of labeling medium (methionine-free DME containing 26 mM Hepes, pH 7.4) at 40°C. The cells were washed twice with 1 ml of labeling medium by brief centrifugation and gentle resuspension, and then incubated for 10 min at 40°C in 0.5 ml of labeling medium. To label, 100 μl of labeling medium containing 500 μCi of [^{35}S]methionine (1,100 Ci/mmol) was added and the incubation continued for 10 min at 40°C. Labeling was stopped by adding unlabeled methionine to a final concentration of 5 mM from a 0.2-M stock. The cells were further incubated for 5 min at 40°C, and then pelleted, washed three times with 0.5 ml of ice-cold growth medium containing 25 mM Hepes, pH 7.4, and resuspended in 0.5 ml of the same medium. Two 40-μl aliquots were removed for normalization, and the remaining cells shifted to a 19.5°C water bath for 2 h to accumulate VSV G in the TGN. Two 150-μl aliquots of cell suspension were pelleted, and gently resuspended in 150 μl of ice-cold Hepes-buffered medium containing either 0 or 5 μg/ml BFA. The cells were shifted to 32°C for 60 min, and then shifted to an ice-water bath, and two 40-μl aliquots were removed from each tube. Immunoprecipitation of total VSV G or VSV G present at the cell surface was then carried out as described previously (Miller and Moore, 1991). Immunoprecipitated VSV G was separated on a 10% SDS-polyacrylamide gel, dried, and exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA); and the amount of VSV G present in each fraction was quantitated using the ImageQuant program (Molecular Dynamics).

Subcellular Fractionation of VSV G

BHK-21 cells, grown on four 10-cm plates, were infected with VSV ts045 and incubated at 39.5°C as described above for cell surface immunoprecipitation. One plate was washed and then shifted to an ice/water bath for control, and the remaining three plates were incubated for 2 h at 19.5°C in 10 ml of buffered growth medium containing 100 μg/ml cycloheximide. All subsequent incubations were in the presence of cycloheximide. One plate was kept in an ice/water bath, and the other two plates were incubated at 32°C in fresh buffered growth medium containing either 0 or 5 μg/ml BFA for 60 min. The plates were shifted to an ice/water bath, and all four plates were washed three times with ice-cold homogenization buffer (10 mM Hepes, pH 7.4, 250 mM sucrose), and lifted from the plates by gentle scraping with a cell scraper in 5 ml of ice-cold homogenization buffer. The cells were pelleted by centrifugation and resuspended in 0.5 ml of homogeniza-

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 μl 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 D_2O buffered with 10 mM Hepes, pH 7.4 (Lodish et al., 1987). The gradients were centrifuged for 3 h at 36,000 rpm at 4°C and 1-ml fractions were collected from the bottom. The protein from 300-μl aliquots of each gradient fraction was precipitated with TCA and separated on a 10% SDS-polyacrylamide gel. The gels were transferred to nitrocellulose, and probed with mAbs to either BiP (Bole et al., 1986), to confirm the localization of the ER, or a mAb specific for the cytoplasmic tail of VSV G (Kreis, 1986) to localize total VSV G. In separate experiments, VSV G was metabolically labeled as described above, and the localization of ^{35}S-VSV G was determined on the gradients under each condition. Similar results were observed in these experiments.

Immunofluorescence and Rhodamine-Transferrin Labeling

BHK-21 cells were plated at 5 × 10^5 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, (Rh-transferrin), the cells were incubated with Rh-transferrin during the accumulation of VSV G in the TGN at 19.5°C as follows. After 30 min at 19.5°C, the media was aspirated from each coverslip and replaced with 2 ml of serum-free buffered growth medium preequilibrated at 19.5°C. After a further 30 min at 19.5°C, the medium was replaced with 2 ml of serum-free buffered growth medium containing 10 μg/ml of Rh-transferrin and the incubation continued for 1 h at 19.5°C. At the end of the incubations 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 immersing for 10 s in −20°C methanol. After fixation and permeabilization the coverslips were washed three times in PBS, incubated for 10 min in 0.1% $NaBH_4$ 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 × 10^5 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 μl of buffered 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 buffered growth medium. Cells were then incubated for an additional hour at 32°C in buffered growth medium containing either 0 or 5 μg/ml BFA. One set of cells was kept at 4°C during this 1-h incubation as control, and a second set of cells was incubated for 1 h at 32°C in buffered 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 s 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 Na_2HPO_4, 20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 g/liter glucose). The cells were then pulse-labeled in 250 μl of buffer A containing 1 mCi/ml [^{35}S]SO_4 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 Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM Na HCO_3, 20 mM Hepes, pH 7.2, 2.2 mM $CaCl_2$, 5.6 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 Instrs., Inc., Fullerton, CA). The pellet was rinsed with 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 by addition of 100 μl of NDET (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 2× 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 phosphorimager screen.

[^{35}S]Sulfate Labeling and Analysis of Constitutive Secretion of Glycosaminoglycan Chains

BHK cells grown on 12-well plates (5 × 10^5 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 [^{35}S]SO_4 in the presence of xyloside, and chased in buffer A containing 5 mM Na_2SO_4 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

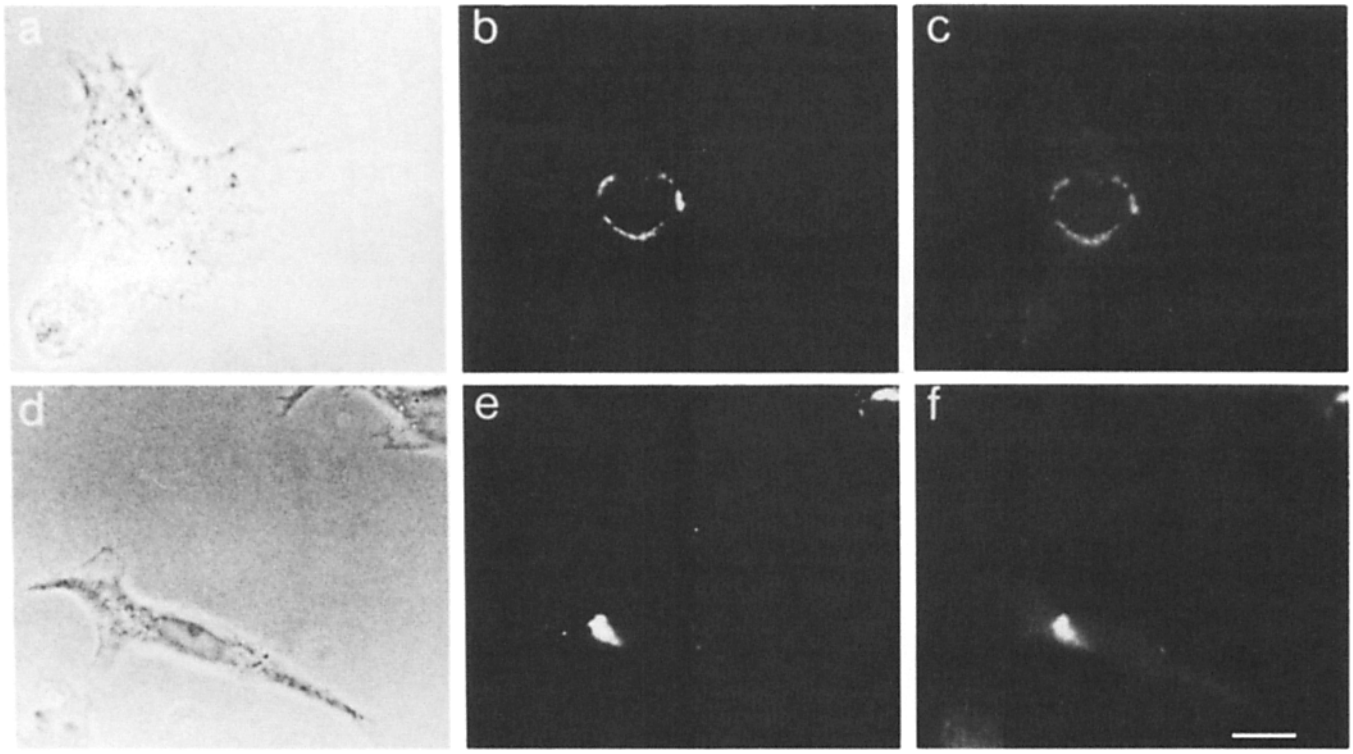


Figure 1. Immunofluorescence localization of VSV G proteins and β-COP in BHK-21 cells that had been infected with VSV ts045 and blocked at 20°C. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, and incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, and again for 2 h in the presence 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.

in media containing cycloheximide to prevent further synthesis of VSV G. The cells were first incubated at 20°C for 2 h to allow the accumulation of VSV G at the TGN. To confirm the intracellular location of the VSV G protein, we performed indirect immunofluorescence and compared the distribution of G protein with the Golgi marker, β-COP. Fig. 1 shows that in cells infected with VSV and incubated at 20°C, VSV G protein is colocalized with β-COP (Fig. 1, *a–f*), which in turn is colocalized with clathrin (data not shown). In most cells, clathrin is concentrated in the TGN (Orci et al., 1985; Tooze and Tooze, 1986), and γ-adaptin, a subunit of the clathrin adaptors of the Golgi complex, is colocalized with the TGN marker TGN-38 (Robinson, 1990). These results are consistent with the EM studies of Griffith et al. (1985) that the 20°C treatment results in accumulation of VSV G proteins in the TGN. To test if transport to the cell surface can occur in the presence of BFA, cells that had been blocked at 20°C were shifted to 32°C in the presence or absence of 5 μg/ml BFA for 1 h. VSV G protein that had reached the cell surface was detected by indirect immunofluorescence of fixed, unpermeabilized cells. Fig. 2 shows that the amount of VSV G protein transported to the cell surface after 15–120 min at 32°C was greatly reduced in the BFA-treated cells compared with untreated controls. To quantitate the extent of this inhibition, VSV G was pulse-labeled for 10 min with [^{35}S]methionine at the end of the 40°C block and then chased at 20°C for 2 h to accumulate the labeled population in the TGN. After shifting to 32°C for 1 h in the presence or absence of BFA, the amount of VSV G on the cell surface was determined by cell surface immunoprecipitation, SDS-PAGE separation of the immunoprecipitated protein, and quantitation using a phosphorimager. As shown in Fig. 3, the extent of transport of VSV G was markedly inhibited by BFA. In the experiment shown, the fraction of VSV G transported to the cell surface during the 32°C chase decreased from 28.4% to 3.8% in the presence of BFA, or by a factor of 7.5. Similar results were obtained when CHO cells were used in these experiments (not shown).

The lack of VSV G protein on the cell surface could be explained by two alternative mechanisms. The obvious explanation is that transport from the TGN to the cell surface is blocked by BFA. Alternatively, VSV G could be transported to the cell surface but, in the presence of BFA, become rapidly internalized after reaching the cell surface, such that very little VSV G is present on the plasma membrane at steady state. The latter possibility was examined by looking at the effect of BFA on VSV G protein after it had reached the cell surface. BHK-21 cells were infected with VSV ts045, incubated for 3 h at 39.5°C to accumulate VSV G in the ER, and then chased for 2 h at 32°C in the presence of cycloheximide to accumulate VSV G at the plasma membrane. The cells were then treated with 5 μg/ml BFA for an additional 1 h at 32°C, and the distribution of VSV G was assayed by indirect immunofluorescence on fixed cells. As shown in Fig. 4 *A*, BFA does not cause redistribution of VSV G from the cell surface; control and BFA-treated cells showed similar

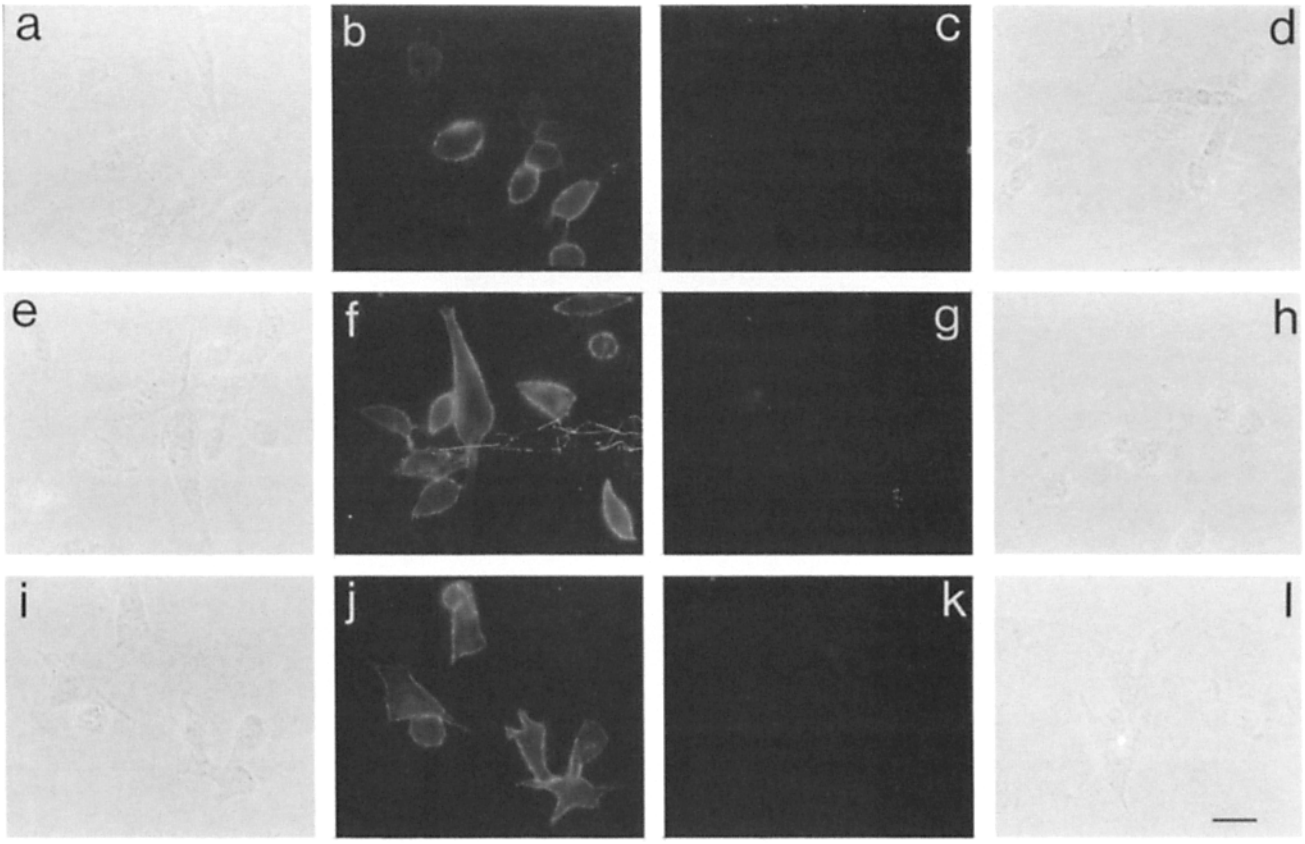


Figure 2. BFA inhibits transport of VSV G from the 20°C compartment to the cell surface. BHK-21 cells were infected and incubated at 20°C to allow accumulation of VSV G proteins in the TGN as in Fig. 1. Cells were then incubated for 15 (*a–d*), 60 (*e–h*), or 120 min (*i–l*) either in the absence (*a*, *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

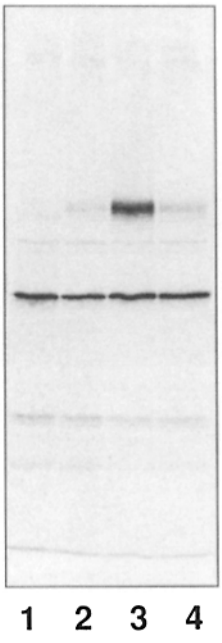


Figure 3. Cell surface immunoprecipitation of VSV G after treatment with BFA. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, pulse-labeled with [^{35}S]-methionine for 10 min at 39.5°C (lane *1*), and then chased for 2 h to accumulate ^{35}S-labeled VSV G in the TGN (lane *2*). The cells were then incubated for 1 h at 32°C in the absence (lane *3*) or presence (lane *4*) of 5 μg/ml BFA. VSV G present on the cell surface was immunoprecipitated (see Materials and Methods) and separated by SDS-PAGE; and ^{35}S-labeled VSV G was detected using a phosphorimager. (Lane *1*) Immediately after pulse-labeling at 39.5°C; (lane *2*) after 2 h accumulation at 19.5°C; (lane *3*) after 1 h of chase at 32°C, −BFA; (lane *4*) after 32°C chase, +BFA. In each case, the amount of total labeled VSV G protein was immunoprecipitated from detergent-extracted cells and used for normalization.

A

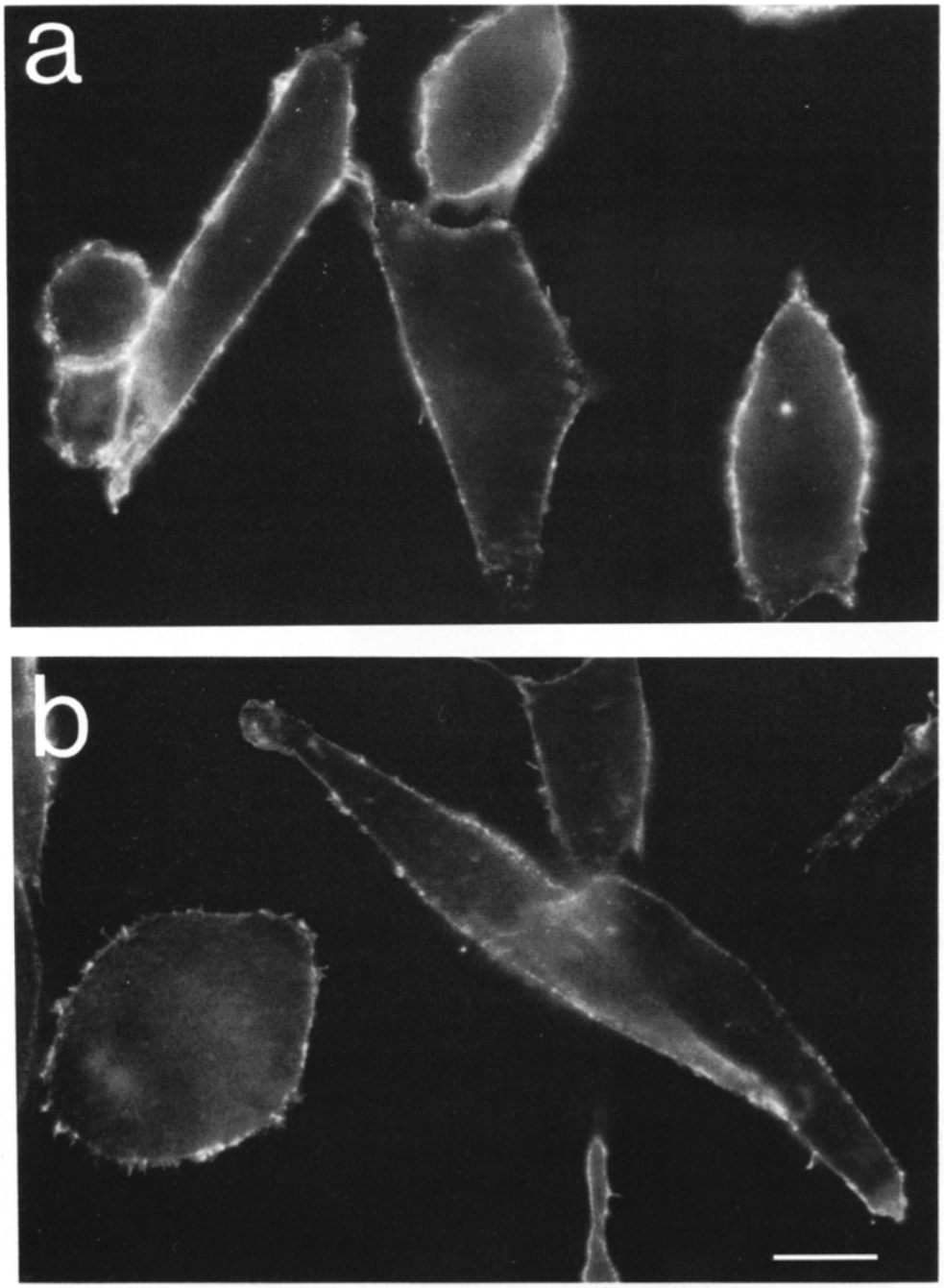
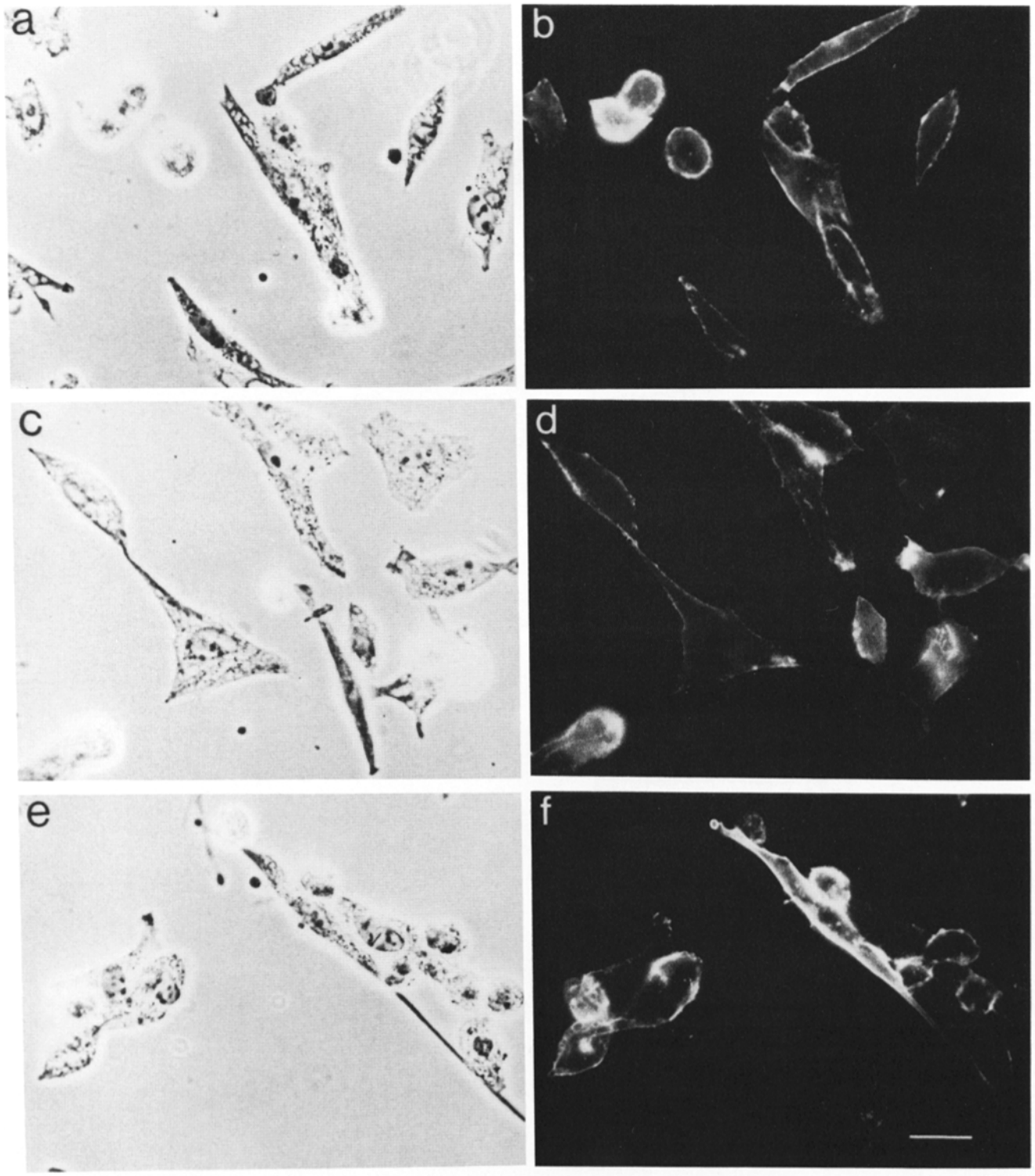


Figure 4. BFA does not lead to loss of VSV G from the cell surface. (*A*) BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 2.5 h at 39.5°C to accumulate VSV G in the ER, and then chased for 1 h at 32°C in the presence of cycloheximide to accumulate VSV G on the plasma membrane. The cells were then incubated for 1 h in the absence (*a*) or presence (*b*) of 5 μg/ml BFA plus cycloheximide. The cells were then fixed and VSV G on the cell surface was detected by indirect immunofluorescence using an antilumenal antibody without permeabilization. (*B*) Same as in *A*, except that after 39.5°C incubation, the cells were first incubated at 20°C for 2 h in the presence of cycloheximide to mimic conditions used in the other experiments. (*a* and *b*) Surface VSV G proteins before the addition of BFA. (*c* and *d*) After a 1-h incubation at 32°C in the absence of BFA, and (*e* and *f*) after a 1-h incubation at 32°C in the presence of BFA. Bars: (*A*) 10 μm; (*B*) 20 μm.

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

B



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/D_2O 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 lumenal 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

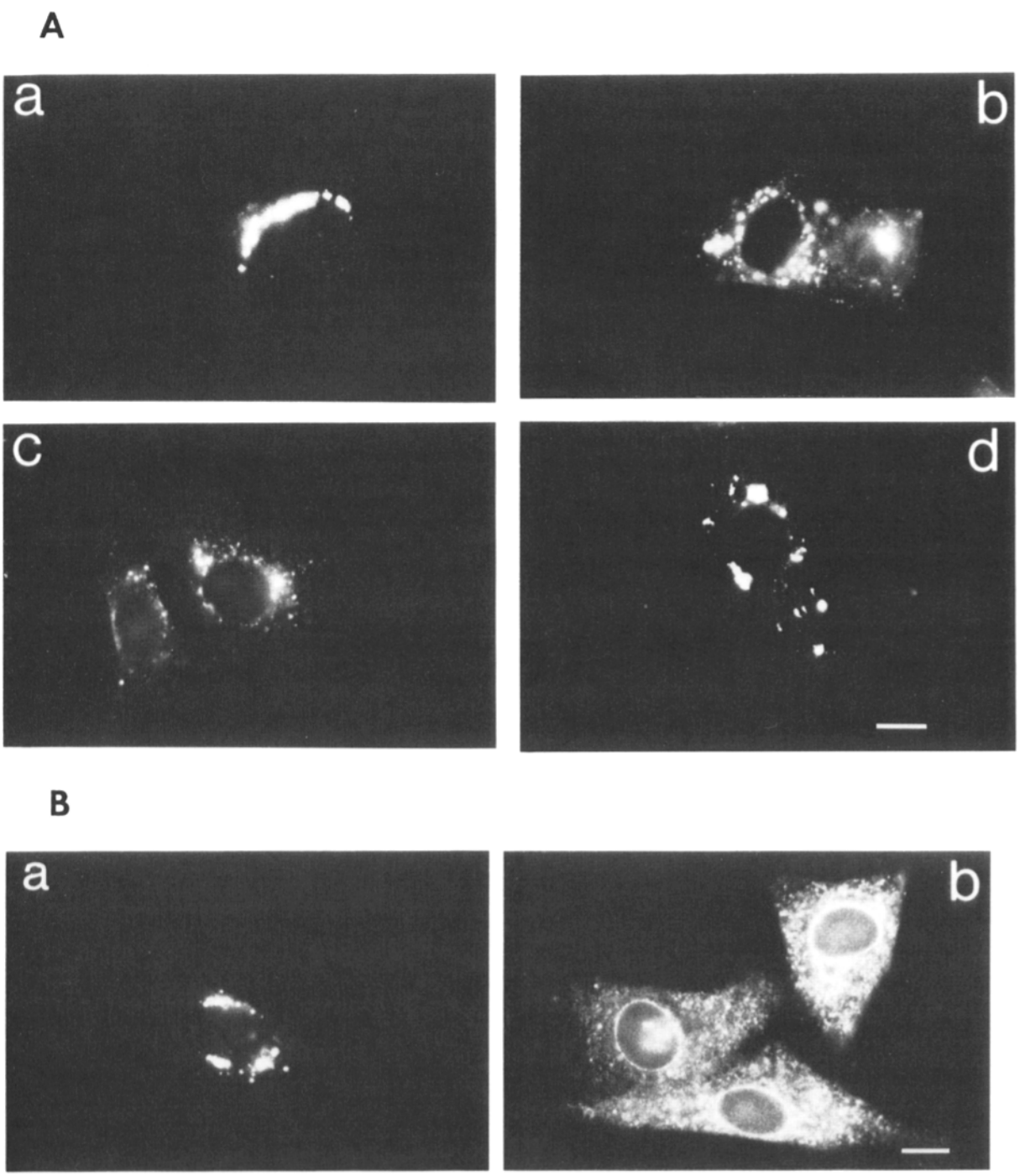


Figure 5. VSV G accumulated in the 20°C compartment does not redistribute to the ER in the presence of BFA. (*A*) BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, and then for 2 h in the presence of cycloheximide at 19.5°C to accumulate 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.

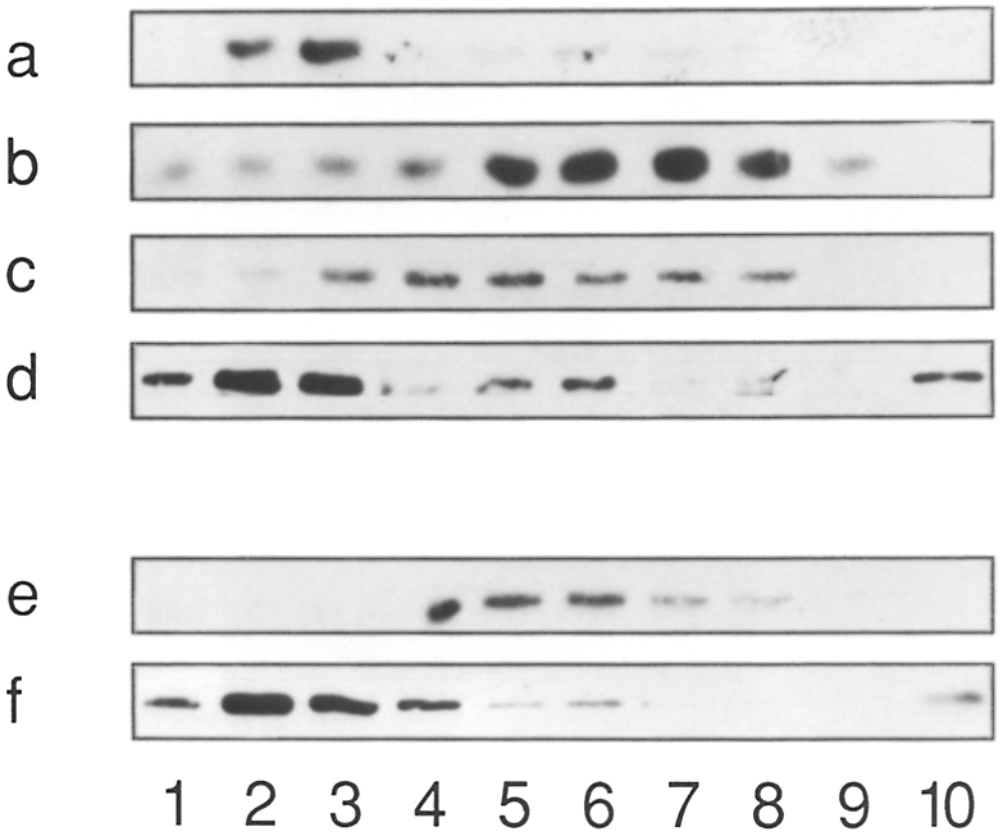


Figure 6. Subcellular fractionation of VSV G and BiP after treatment with BFA. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER (*a*), and again for 2 h at 19.5°C in the presence of cycloheximide to accumulate VSV G in the TGN (*b*). 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/D_2O 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 mannose-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

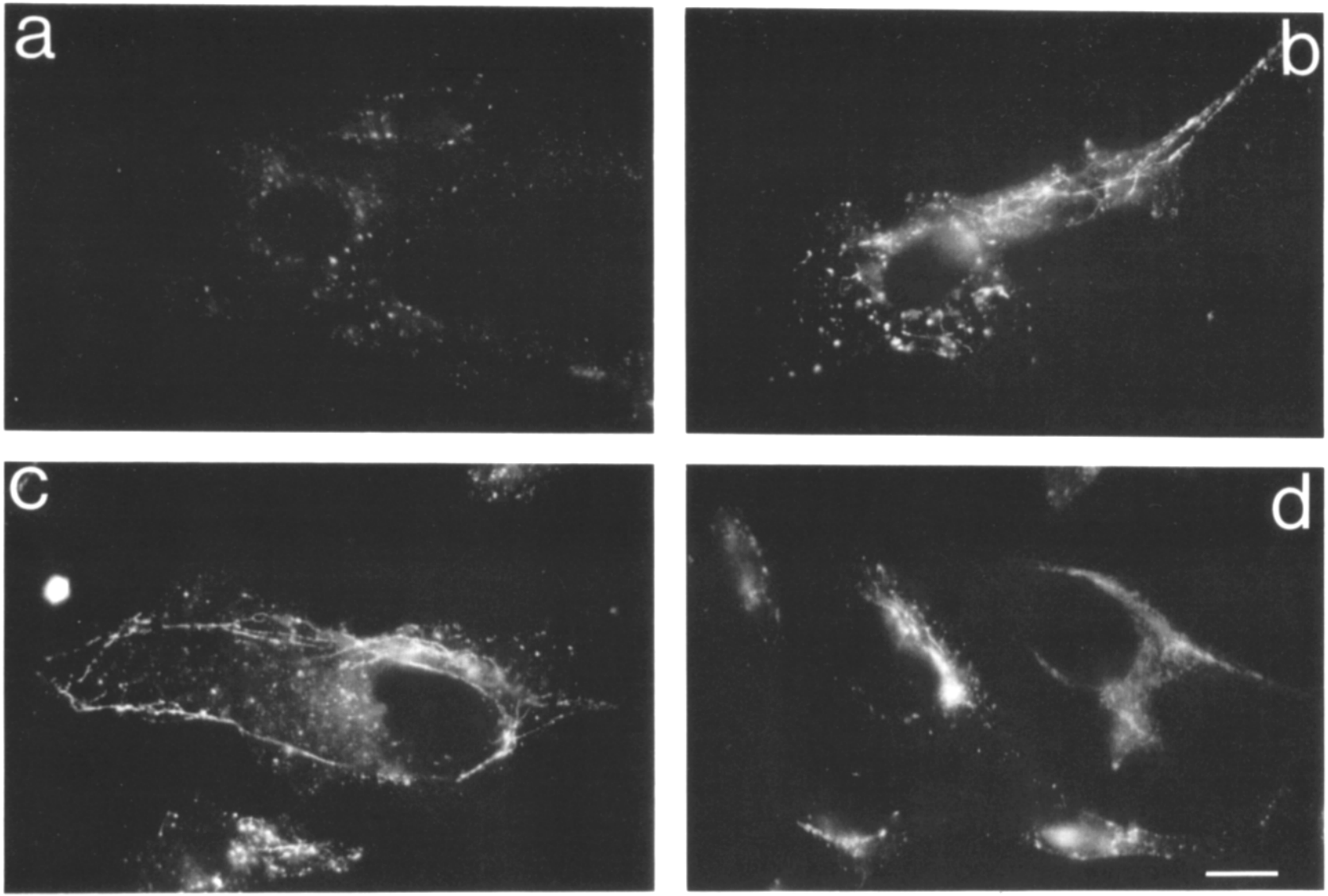


Figure 7. BFA causes redistribution of early endosomes into tubular structures. BHK-21 cells were incubated for 1 h at 37°C in serum-free medium containing 10 μg/ml Rh-transferrin; BFA was added to a final concentration of 5 μg/ml; and the incubation was continued for 0 (*a*), 15 (*b*), 60 (*c*), or 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.

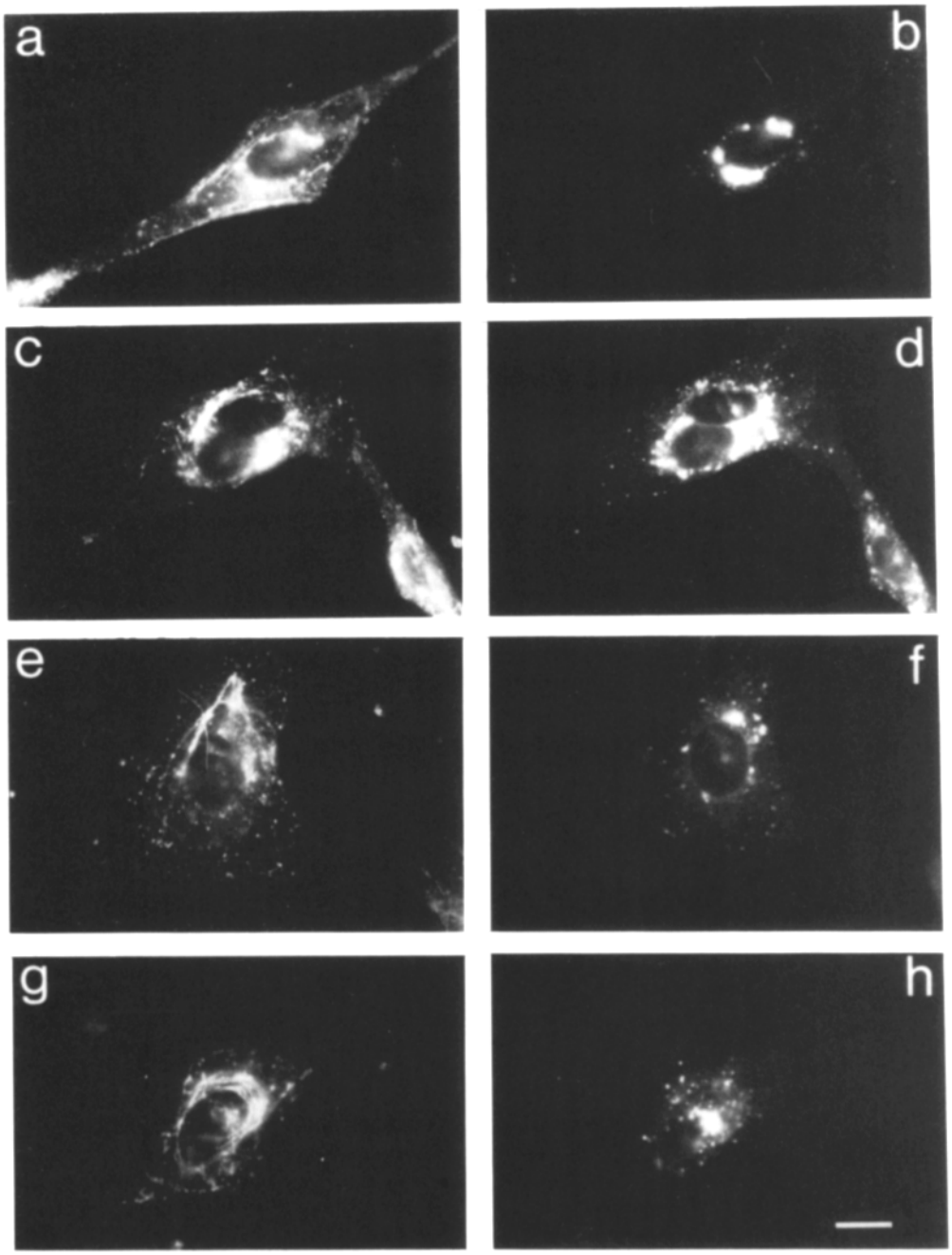


Figure 8. Double labeling of internalized Rh-transferrin and VSV G after treatment with BFA. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 3.5 h at 39.5°C to accumulate VSV G in the ER, and then incubated for 2 h in the presence of cycloheximide at 19.5°C to accumulate VSV G in the TGN. The early endosomal compartment was labeled with Rh-transferrin during the 19.5°C incubation as described in Materials and Methods. The cells were then chased at 32°C for 15 (*a* and *b*), 60 (*c* and *d*), and 120 min (*e–h*) in the presence of 5 μg/ml BFA and 10 μg/ml Rh-transferrin plus cycloheximide. The cells were fixed and permeabilized, and internal VSV G was detected by indirect immunofluorescence using a fluorescein-conjugated secondary antibody (*b*, *d*, *f*, and *h*). Internal Rh-transferrin was visualized using rhodamine optics (*a*, *c*, *e*, and *g*). 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

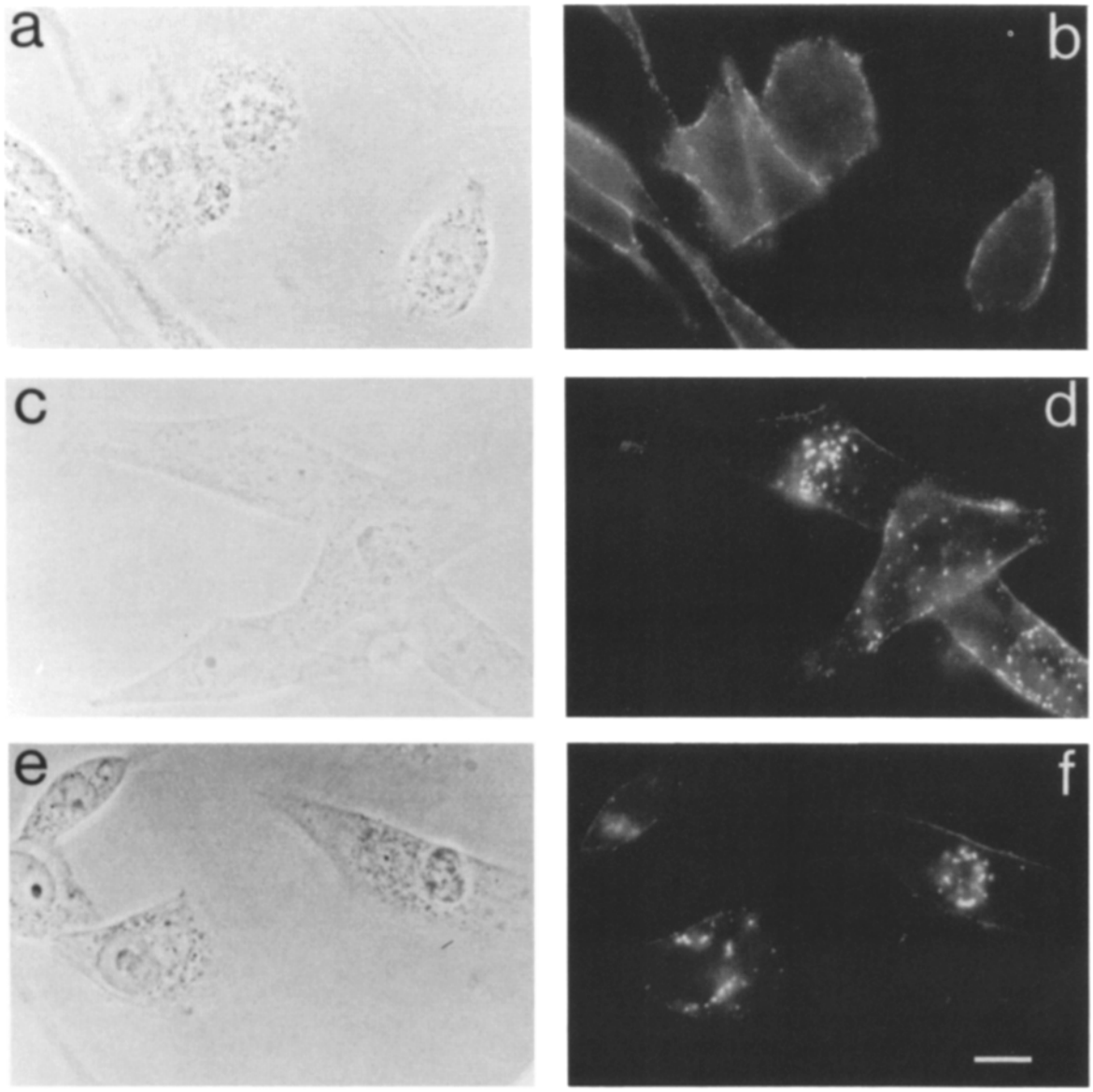


Figure 9. BFA does not block endocytosis of cell surface VSV G protein cross-linked with antibodies. BHK-21 cells were infected with VSV ts045 for 45 min at 32°C, incubated for 2.5 h at 39.5°C to accumulate VSV G in the ER, and then incubated for 1 h in the presence of cycloheximide at 32°C to accumulate 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 $[^{35}S]SO_4$, 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 $[^{35}S]SO_4$ 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%

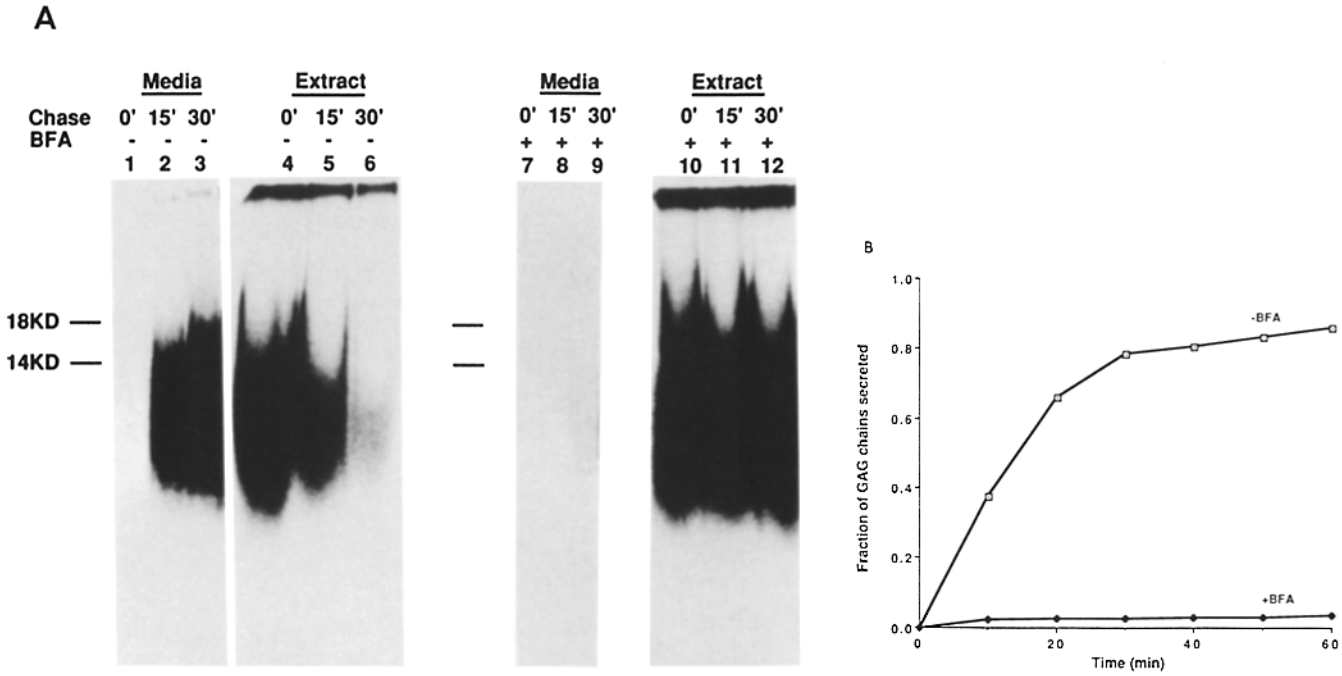


Figure 10. BFA blocks secretion of $[^{35}S]SO_4$ GAG chains via the constitutive pathway. (*A*) BHK-21 cells grown in 12-well plates were preincubated with 0.5 mM xyloside for 30 min at 37°C, and pulse-labeled with $[^{35}S]SO_4$ for 2 min. The cells were then chased in medium containing 5 μg/ml BFA 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.

of GAG chains secreted during a 1-h chase period. GAG chains that were not secreted in BFA were quantitatively recovered in the cell extracts. These results indicate that BFA inhibits export of both soluble and membrane-bound molecules from late Golgi compartments.

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 $[^{35}S]SO_4$ for 5 min, and then chased in the presence of BFA for 1 h to allow the accumulation of ^{35}S-labeled secretogranin in regulated secretory vesicles. The cells were then depolarized by incubation for 15 min in medium containing high K^+ Ca^{2+} 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 TGN via both 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 ^{35}S-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

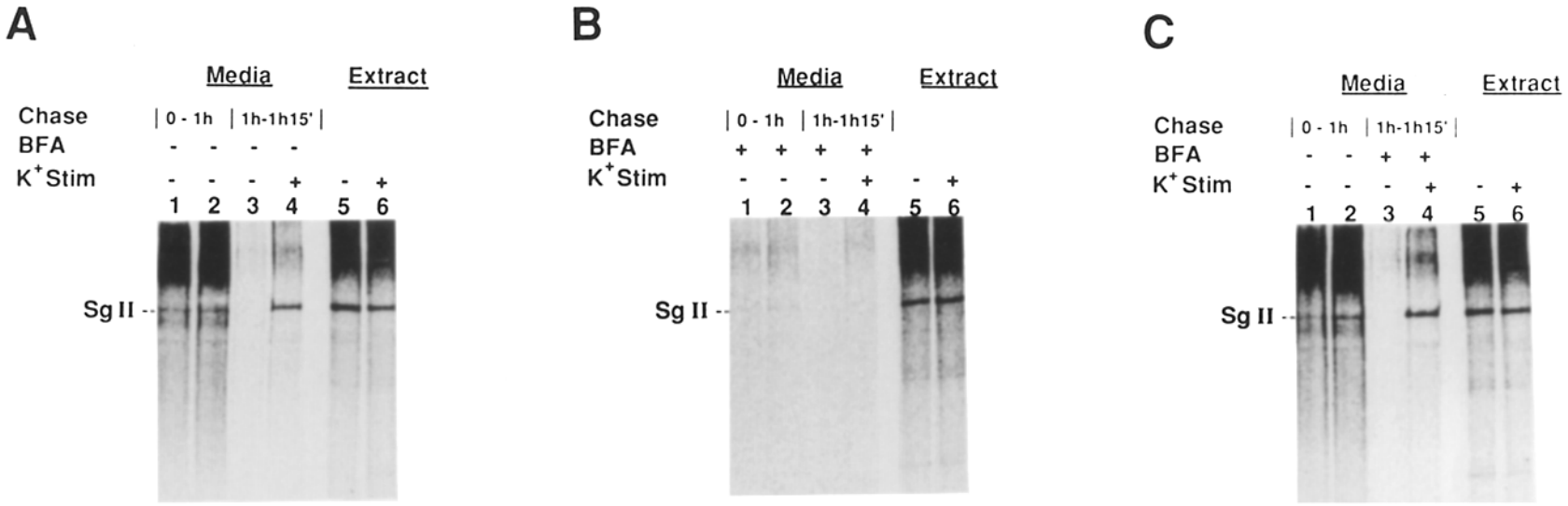


Figure 11. Entry of $[^{35}S]SO_4$ secretogranin II into the regulated secretory pathway is inhibited by BFA. PC12 cells on a 12-well plates were pulsed-labeled with $[^{35}S]SO_4$ for 5 min and chased in unlabeled medium containing low K^+ for 1 h to allow packaging of labeled secretogranins into storage 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 potently 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 mixing 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 coatomer 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., 1991*b*; 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 of export from the TGN is rapid (well within 15 min, data not shown), as is the dissociation of β-COP. An alternative interpretation proposed earlier is that the coatamer complex may function as a dynamic framework, or scaffold, to maintain the structure of the Golgi complex and the selectivity of membrane budding and/or fusion (Duden et al., 1991*a*). At the present time, it is not easy to determine which of these two roles the coatomer plays. However, if it functions to provide membrane selectivity, it is not easy to explain why dissociation of β-COP from the TGN does not cause it to coalesce with other Golgi cisternae and the ER (see below).

A surprising finding is that BFA prevents secretion via the constitutive and the regulated secretory pathways. This is not expected if β-COP is the target of BFA, since clathrin has been found to be associated with incipient regulated secretory granules (Orci et al., 1985) and has been generally assumed to be the coat for budding regulated secretory granules. One possibility is that β-COP is not the direct target for BFA. Rather, the target(s) for BFA may be components essential for assembly of a variety of different coats. Indeed, it was recently reported that BFA causes selective dissociation of clathrin and adaptors from the Golgi but not from the plasma membrane in VERO cells and MDCK cells (Robinson and Kreis, 1992; Wong, D. H., and F. M. Brodsky, unpublished results). Similar results were obtained in BHK cells (Miller, S., and H. P. Moore, unpublished observations). Furthermore, Ktistakis et al. (1991) used a BFA-resistant cell line, PtK_1, in cell fusion studies to show that the target for BFA is a nondiffusable factor. β-COP, on the other hand, appears to be in dynamic equilibrium with the Golgi membranes: a large fraction of the coatomer complex is found in the soluble fraction, where it comprises ~0.2% of total cytosolic protein (Waters et al., 1991); it dissociates from the membrane when cells are depleted of ATP (Donaldson et al., 1991); and GTPγS, which blocks intracellular transport, prevents its dissociation by BFA (Donaldson et al., 1991). Thus, it is more likely that the targets of BFA are membrane-associated proteins involved in the assembly of coats, such as the yeast SEC12 gene product, an integral membrane glycoprotein (Nakano et al., 1988) that interacts with the low molecular weight GTP-binding protein, Sarlp (Nakano and Muramatsu, 1989) in the formation of ER vesicles (d'Enfert et al., 1991). Further work will be necessary to determine if the inhibitory effects of BFA on export via the constitutive and the regulated pathway is due to dissociation of nonclathrin, and clathrin coats form the TGN, respectively. It should be noted that our conclusion of blockage to regulated secretory pathway is based on the assumption that tyrosine sulfation occurs at least in part in the TGN. This is likely to be the case since it has been shown that tyrosine sulfation is the last modification before exit from the TGN, after sialylation (Baeuerle and Huttner, 1987), and that tryosylprotein sulfotransferase is itself sialylated (Niehrs and Huttner, 1990). Since a significant fraction of the sialyltransferase resides in the TGN (Roth et al., 1985; Berger et al., 1987), it is reasonable to assume that this compartment represents a major site for tyrosine sulfation.

Our data, in conjunction with earlier studies, suggest that the exo/endocytic compartments may be composed of two similar but distinct membrane systems; the ER/*cis*-/medial-/*trans*-Golgi system, and the TGN/cell surface/endosomal system. Both systems are similarly affected by BFA; i.e., for-

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, it 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 coatomer 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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