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Differential Effects of Brefeldin A on Transport of Secretory and Lysosomal Proteins*

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Brefeldin A (BFA) rapidly blocks anterograde exocytotic transport through the Golgi complex. Sustained retrograde traffic induced by brefeldin A causes redistribution of constituents of the Golgi, but not the trans-Golgi network (TGN), to the endoplasmic reticulum (ER). In the present study on HepG2 cells, we have observed a differential effect of BFA on transport from the TGN of two soluble proteins: α_1-antitrypsin as a representative of secretory proteins and cathepsin D as a prototype of lysosomal enzymes. The Golgi complex of HepG2 cells is sensitive to BFA, as within minutes after its addition nearly all activity of three resident Golgi enzymes was recovered in the ER as monitored by cell fractionation on sucrose density gradients. In accordance with this, "high mannose"-glycosylated α_1-antitrypsin was retained in or transported back to the ER. "Complex"-glycosylated α_1-antitrypsin was neither secreted into the medium nor transported back to the ER. Most of it was retained in vesicles with the buoyant density of Golgi. These vesicles contained the fluid phase endocytotic marker horseradish peroxidase when this was added to the culture medium prior to the BFA, suggesting that the vesicles derived from the TGN. After BFA addition, the compartment became inaccessible to endocytosed horseradish peroxidase. In contrast to blocking transport of complex α_1-antitrypsin, BFA did not affect processing of newly synthesized complex-glycosylated procathepsin D (53 kDa) to the mature 31-kDa form. Neither did it interfere with processing of endocytosed procathepsin D. That the mature cathepsin D had indeed reached the lysosomes was verified by Percoll density gradient fractionation. In conclusion, in HepG2 cells, BFA induces two blocks in the secretory pathway: one at the level of the ER-Golgi juncture and the other in the TGN. In contrast, transport from the Golgi complex to the lysosomes and from the plasma membrane to the lysosomes continued.

The vacuolar system transports and directs membrane constituents and contents from the site of synthesis, the rough endoplasmic reticulum (ER),[1] via the Golgi complex to different destinations inside and outside cells. Although the primary role of the ER is insertion into or translocation across the membrane and core glycosylation of newly synthesized proteins, the Golgi complex contains the enzymes for final oligosaccharide processing of both glycoproteins and glycolipids.

Traffic from ER to Golgi and between individual Golgi cisternae takes place via non-clathrin-coated vesicles that constitutively transport content and membrane components destined for the cell surface. Proteins can leave the ER only if they have acquired the proper spatial conformation and appropriate oligomeric structure (4, 12). The site of exit is believed to be the transitional elements that bud out into transitional vesicles, also named the "salvage" or intermediate compartment (19, 22, 27). In co-operation with the cis-Golgi, it is thought to form the sorting compartment at the entrance of the Golgi complex. At the trans-face of the Golgi, sorting has been assigned to a reticulum of tubules and fenestrated cisternae with associated coated and smooth surfaced vesicles. This organelle, referred to as trans-Golgi network (TGN), has a central role in regulating traffic within the distal part of the vacuolar membrane system (2, 9, 10). In this way, the Golgi complex takes part in two distinct cycling pathways in which sorting occurs: the first one is in the ER-Golgi region with vesicular transport between elements of the ER, the intermediate compartment, and the Golgi stack and the second is the juncture between the endocytic and exocytic pathways where membrane constituents recycle between the TGN, endosomes, and plasma membrane. Whereas components are continuously delivered from the cis-compartments into the TGN, it is unclear whether vesicular traffic in the reverse direction occurs.

In a number of cell types, return traffic from Golgi to ER is induced by the drug brefeldin A (BFA). It causes the Golgi stack to disassemble and resident Golgi proteins to be transported back into the ER, thus interfering with forward (bulk-phase) transport (15, 16, 33). The transport of newly synthesized membrane proteins and secretory proteins is completely inhibited in the presence of BFA (7, 20). BFA appears to induce fusion of the Golgi-derived vesicles with the ER resulting in an ER-Golgi compartment in which enzymatic components of the two compartments are intermixed (32). Recently, it has been shown that in the presence of BFA, β-COP, one of the coat proteins of non-clathrin-coated vesicles, dissociates from the Golgi membrane (6). This effect may be the primary cause of the disintegration of the Golgi complex.

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[1] The abbreviations used are: ER, endoplasmic reticulum; BFA brefeldin A; MP-R, mannose phosphate receptor; MEM, Eagle's minimal essential medium; TGN, trans-Golgi network; Endo H, endo-β-N-acetylglucosaminidase H; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DAB, 3,3′-diaminobenzidine tetrahydrochloride.

It has been observed that proteins localized within or beyond the TGN do not return to the ER in the presence of BFA (2, 23, 26). However, BFA does also affect the second recycling pathway of the vacuolar system (17, 35). Addition of BFA resulted in tubule formation and fusion of the TGN and endosomes. This hybrid system remained functional in recycling of endocytosed membrane proteins to the plasma membrane (2, 26). Light microscopic studies suggested that endocytic transport to lysosomes is impaired in some (17) but not in other cell types (35).

To further define the effect of BFA on protein transport out of the TGN, we studied the transport of α_1-antitrypsin as a representative of secretory proteins and of cathepsin D as a prototype lysosomal enzyme in HepG2 cells. Although most of the activity of three glycosyltransferases in the presence of BFA moved to organelles with a buoyant density typical for rough ER, virtually all of the complex glycosylated form of the secretory protein α_1-antitrypsin remained in vesicles with a relatively low buoyant density. Secretion of α_1-antitrypsin from this compartment no longer occurred. In contrast, transport of the complex glycosylated form of procathepsin D to lysosomes proceeded.

EXPERIMENTAL PROCEDURES

Materials—HepG2 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (28). Fresh culture medium was added 1 day before the experiments. BFA (a gift from Sandoz, AG, Basel, Switzerland) was dissolved in ethanol and used in concentrations of 1, 5, or 10 μg/ml. Pure solvent was added to control media. Rabbit antisera against human α_1-antitrypsin and albumin were from Dakopatts, Denmark. Antiserum against cathepsin D was raised in rabbits against enzyme purified from human placenta. *N*-6-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-aminocaproyl-ceramide was synthesized as before (34). Culture media and solutions were obtained from Gibco (Glasgow, United Kingdom); bovine serum albumin, fraction V, was purchased from Sigma. All other chemicals and solvents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Metabolic Labeling and Analyses—Nearly confluent HepG2 cells grown on 35-mm culture dishes were pulse-labeled in methionine-free MEM containing [^{35}S]methionine (Tran^{35}S-label, 1.85 MBq/ml, 40 TBq/mmol, ICN) and chased in the presence of MEM-containing fetal bovine serum and unlabeled methionine. For cell fractionation on sucrose gradients, the cells were swollen in a low salt buffer (10 mM Hepes-NaOH, 15 mM KCl, 1.5 mM $MgCl_2$, pH 7.2) at 0 °C, scraped from the dish, and homogenized in a tight-fitting Dounce homogenizer (31). The postnuclear supernatant was layered on top of a linear gradient (0.7–1.5 M sucrose in 1 mM EDTA, 10 mM Hepes, pH 7.2), and the 11-ml gradient was centrifuged in a Spinco SW-41 rotor for 3 h at 38,000 rpm. Equal fractions were collected. For cell fractionation on Percoll gradients, the cells were passed 15 times through two 30-gauge needles (30G1/2) connected by 100 mm of polythene tubing of 0.28-mm inner diameter in 0.25 M sucrose, 1 mM EDTA, and 10 mM Hepes, pH 7.2. Nuclei and intact cells were removed by centrifugation for 10 min at 300 × *g*, and the postnuclear supernatant was layered on top of 7.8 ml of 30% Percoll in the same buffer and centrifuged in a Beckman Ti-50 rotor for 25 min at 100,000 × *g*. For direct immunoprecipitation, cells were lysed in 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline (PBS). [^{35}S]Methionine-labeled proteins were quantitatively immunoprecipitated from cell lysates or gradient fractions (31) and analyzed by SDS-PAGE. The gels were fluorographed, and the fluorograms were scanned with a LKB laser-equipped microdensitometer for quantitation. Endo H treatment was performed as described previously (31). Following appropriate labeling and immunoprecipitation, the samples were divided in two equal portions that were incubated for 18 h at 37 °C in 0.25 M citrate buffer, pH 6.0, containing 1% SDS and 1 mM phenylmethylsulfonyl fluoride in the absence or presence of 5 μg/ml Endo H. Thereafter, the samples were precipitated by acetone and analyzed by SDS-PAGE and fluorography.

Enzyme Assays and Western Blotting—The activity of UDP-glucose:ceramide glucosyltransferase (EC 2.4.1.80) was assayed in gradient fractions obtained after fractionation of a 9-cm culture dish using the fluorescent precursor *N*-6-(7-nitro-2,1,3-benzoxadiazol-4-yl)-aminocaproyl-ceramide (13). Galactosyltransferase was assayed as described previously using ovalbumin as acceptor (31). Sialyltransferase was assayed in the gradient fractions using asialofetuin as acceptor (21). Western blotting was performed as described previously (30).

DAB Cytochemistry—DAB cytochemistry has been described previously (30). Cells (6-cm dishes) were incubated in 1.5 ml of MEM containing 3 mg/ml horseradish peroxidase. Following horseradish peroxidase uptake and [^{35}S]methionine labeling, the cells were washed on ice with MEM buffered with 10 mM Hepes, pH 7.2 (3 × 10 min), and PBS containing 1 mM EDTA and subsequently treated with proteinase K (0.5 mg/ml in PBS, 1 mM EDTA) for 60 min at 0 °C to release the cells from the dish and to remove nonspecifically bound extracellular horseradish peroxidase. The digestion was terminated by washing the cells in PBS containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride by centrifugation (5 min, 300 × *g*). The cell suspension was divided into two equal portions and incubated in 0.35 ml of PBS containing 1 mM EDTA, 0.3 mg/ml DAB and in the presence or absence of 0.06% H_2O_2 on ice for 45 min in the dark. Cells were then washed with PBS, 1 mM EDTA, and lysed in sample buffer for SDS-PAGE and Western blotting.

RESULTS

Effect of BFA on the Distribution of Golgi Enzymes—Most of the Golgi functions have been assigned to one of the four subcompartments: intermediate compartment, cis-Golgi, trans-Golgi, and TGN. BFA has remarkable effects on the structure and identity of the Golgi complex. We have treated HepG2 cells with BFA to determine how the distribution of both soluble and membrane proteins in the different subcompartments were affected (Fig. 1). UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase has frequently been used as marker enzyme for the two or three transmost cisternae of the Golgi stack (25). ER was recovered in fractions 3–5 as was determined by the presence of "high mannose" α_1-antitrypsin from [^{35}S]methionine pulse-labeled cells (not shown). Fig. 1 shows that after BFA treatment ±70% of UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase enzyme activity fractionated at the density of the rough ER. This was also the case for sialyltransferase, another trans-Golgi enzyme. As we used asialofetuin as an acceptor for acetylneuraminic acid addition, it is likely that in the enzyme assay used in Fig. 1 both α2,3- and α2,6-sialyltransferase were measured (21). Approximately 30% of both UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase and sialyltransferase remained at its original density and thus seemed unaffected by BFA. The distribution of a Golgi enzyme involved in the synthesis of membrane glycolipids, UDP-glucose:ceramide glucosyltransferase, was also affected by the BFA treatment (Fig. 1), but a portion of the latter enzyme cofractionated with BFA-resistant UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase- and sialyltransferase-containing vesicles. Thus, in HepG2 cells, at least three Golgi markers redistributed in part toward the ER in the presence of BFA.

Characterization of a Second BFA Block in the Secretory Route—To determine whether BFA affects transport between the Golgi complex and the cell surface, the biosynthetic processing and secretion of α_1-antitrypsin were measured. We used α_1-antitrypsin as a prototype, secretory protein as its precursor (high mannose), and mature (complex) configurations can easily be separated by SDS-PAGE and quantitated. Cells were labeled with [^{35}S]methionine for 35 min, yielding labeling of the entire secretory route with ^{35}S-labeled α_1-antitrypsin. Next, the cells were chased for various periods of time in the presence of a high concentration of BFA (10 μg/ml), ensuring an instantaneous effect of the drug (Fig. 2). The redistribution of the transferases depicted in Fig. 1 occurred within 5 min. In the absence of BFA all high mannose α_1-

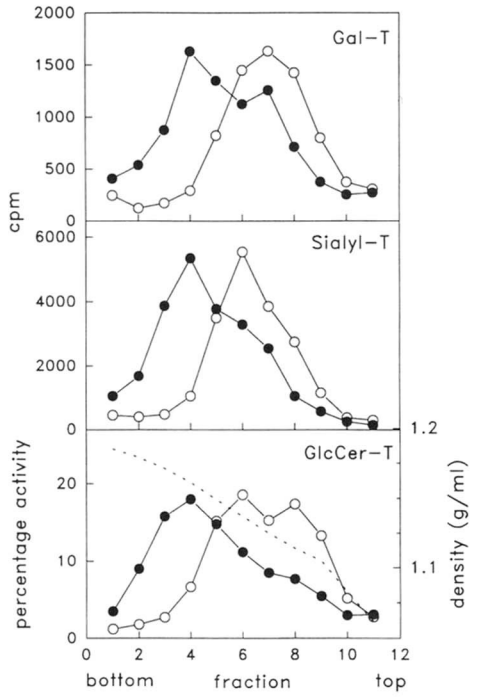

FIG. 1. **Effect of BFA on the subcellular distribution of Golgi enzymes.** HepG2 cells were incubated in normal growth medium for 60 min in the presence (●) or absence (○) of 1 μg/ml BFA. The cells were homogenized, and the postnuclear supernatant was fractionated on 0.7–1.5 M linear sucrose gradients. The *upper* (UDP-galactose β-D-*N*-acetylglucosamine β,1–4 transferase (*Gal-T*)) and *middle* (sialyltransferase (*Sialyl-T*)) *panels* represent typical experiments. Curves of the *lower panel* (UDP-glucose:ceramide glucosyltransferase (*GlcCer-T*)) are the mean of four (control) and six (BFA) experiments; total activity of UDP-glucose:ceramide glucosyltransferase in the cell homogenates after BFA were on average 1.4 times that of control cell homogenates. Densities of the gradients fractions are indicated by the *dotted line* in the *bottom panel.*

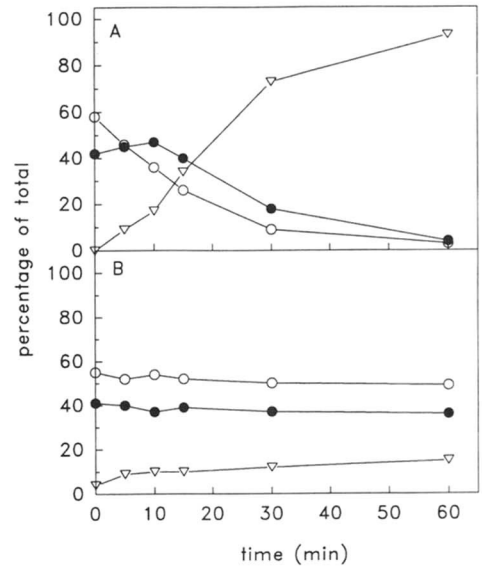

FIG. 2. **Effect of BFA on the processing and transport of α_1-antitrypsin.** Cells were labeled for 35 min with [^{35}S]methionine and chased for the time indicated, either in the presence (*B*) or absence (*A*) of 10 μg/ml of BFA. This labeling time was chosen to have both high mannose and complex forms of α_1-antitrypsin labeled at the beginning of the chase period. Aliquots of chase media and lysed cells were taken, and α_1-antitrypsin was immunoprecipitated and analyzed by SDS-PAGE. The gels were fluorographed, and ^{35}S-labeled α_1-antitrypsin was quantitated by densitometry. ○, high mannose; ●, complex cell-associated; ▽, secreted ^{35}S-labeled α_1-antitrypsin.

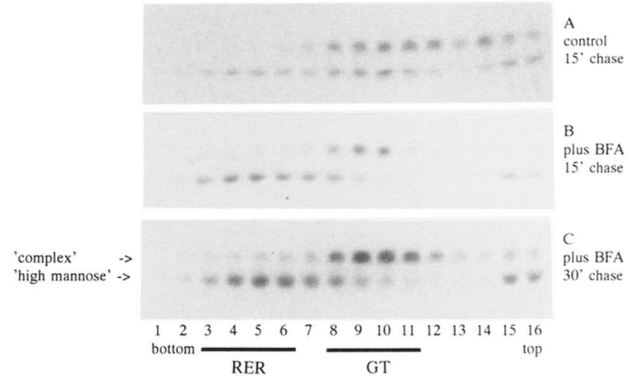

FIG. 3. **Effect of BFA on the subcellular distribution of complex α_1-antitrypsin.** Cells were labeled as described in the legend to Fig. 2 and chased for 15 (*A–B*) or 30 min (*C*) in the presence (*B–C*) or absence (*A*) of BFA. Postnuclear supernatants of cell homogenates were fractionated on 0.7–1.5 M sucrose gradients. α_1-Antitrypsin was immunoprecipitated from the fractions and analyzed by SDS-PAGE. The rough ER fractions (*RER*) were identified by a 5-min labeling, after which labeled α_1-antitrypsin was only detected in fractions 3–6; the Golgi-containing fractions (*GT*) were identified by measuring the galactosyltransferase enzyme activity.

antitrypsin was converted into complex enzyme and the secretion of pulse-labeled α_1-antitrypsin was virtually complete after 60 min (Fig. 2*A*). If BFA was present during the chase (Fig. 2*B*), both transport and processing were affected: (i) within 5 min, secretion of complex α_1-antitrypsin completely stopped and (ii) no further conversion of high mannose to complex form took place.

Next, we tested whether complex α_1-antitrypsin, which in the presence of BFA was no longer secreted, had been transported to the ER. Other studies (5, 15) would predict that, in the presence of BFA, medial and trans-Golgi constituents would be transported to the ER, whereas TGN and transport vesicles to the plasma membrane would persist. Complex antitrypsin would be expected to behave accordingly. To measure the intracellular distribution of complex α_1-antitrypsin in BFA-treated cells, we labeled cells for 35 min and chased for 15 or 30 min either in the presence or absence of BFA. The localization of the ER compartment on sucrose density gradients was identified by the presence of high mannose α_1-antitrypsin after a short pulse labeling with [^{35}S] methionine (indicated as *RER*). After a 15-min chase in the absence of BFA, high mannose α_1-antitrypsin was distributed over ER and the Golgi fractions (Fig. 3*A*), whereas complex α_1-antitrypsin localized to trans-Golgi vesicles (fractions 8–11). After a chase in the presence of BFA, the bulk of the complex form remained at the density of "Golgi" vesicles (Fig. 3, *B* and *C*, fractions 8–11). However, high mannose α_1-antitrypsin became partly redistributed to ER fractions. If the experiment was repeated for albumin, the same phenomena were observed; part of the protein remained in the Golgi-like vesicles in the presence of BFA (not shown). Thus, upon BFA addition, the complex form became trapped in a Golgi or post-Golgi compartment from where neither forward nor backward protein traffic occurred.

Endocytosed Horseradish Peroxidase Is Transported to α_1-Antitrypsin Containing Compartments—Chege and Pfeffer (2) have shown that no *membrane* proteins present in the TGN move back to the ER in the present of BFA. This is consistent with our finding that, upon addition of BFA, the majority of complex α_1-antitrypsin accumulated in a Golgi-like compartment. To further characterize this compartment, we made use

of our previous finding that endocytic and exocytic pathways are connected in the TGN (24, 29). Fluid phase-endocytosed horseradish peroxidase was used to monitor this connection. The peroxidase activity was utilized to polymerize DAB specifically in the horseradish peroxidase-containing vacuoles with the consequent cross-linking of the protein contents to DAB polymers (29, 30). Endocytosed horseradish peroxidase that is introduced into the TGN thus would be expected to cross-link complex α_1-antitrypsin. Indeed, 40% of the complex-type α_1-antitrypsin was lost due to cross-linking to DAB polymers, whereas the high mannose form was unaffected (Fig. 4*A*), indicating that endocytosed horseradish peroxidase entered the secretory pathway at the TGN. Next, we tested whether horseradish peroxidase that entered the TGN could be detected in the rough ER upon BFA treatment of the cells. To answer this question we introduced horseradish peroxidase into the TGN before BFA was added to the culture medium; cells were first incubated in medium containing 3 mg/ml horseradish peroxidase. After 30 min, BFA was added and, after another 15 min, [^{35}S]methionine was added to the medium and the incubation was continued for 45 min to label newly synthesized proteins (Fig. 4*B*). Colocalization of horseradish peroxidase with α_1-antitrypsin, localized in the ER, was monitored by measuring the degree of cross-linking of α_1-antitrypsin to DAB polymers upon incubation of the cells with DAB/H_2O_2. Transport of α_1-antitrypsin out of the ER was impeded while Golgi enzymes including glycosyltransferases were introduced in the ER. As the latter enzymes induced only partial processing of α_1-antitrypsin oligosaccharides, the appearance after SDS-PAGE of the high mannose band changed (Fig. 4*B*). In the presence of BFA, no high mannose α_1-antitrypsin nor partially complex α_1-antitrypsin were affected by DAB cytochemistry. We conclude that no detectable quantity of fluid phase-endocytosed horseradish peroxidase was transported from the TGN to the ER during BFA treatment.

To answer the question of whether fluid phase-endocytosed horseradish peroxidase can gain access to the compartment in which complex α_1-antitrypsin is trapped during BFA treatment, experiments as depicted in Fig. 4, *C-E*, were performed. No cross-linking of any α_1-antitrypsin was observed if the addition of BFA preceded the addition of horseradish peroxidase (Fig. 4*D*), whereas 40% of complex but none of the high mannose α_1-antitrypsin was cross-linked if BFA was omitted (Fig. 4*C*). In contrast, 30–40% of complex α_1-antitrypsin was cross-linked if BFA was administered after horseradish peroxidase (Fig. 4*E*). Taken together, the results imply that the complex α_1-antitrypsin is transported through a compartment from which, in the presence of BFA, neither transport back to the rough ER nor exocytic transport occurred; this compartment is accessible to the endocytic marker horseradish peroxidase in the absence but not in the presence of BFA.

Cathepsin D Is Proteolytically Processed and Transported to Lysosomes in the Presence of BFA—Once established that BFA blocks transport of mature secretory proteins at or after the TGN, we studied whether transport from TGN to lysosomes is prohibited as well. In HepG2 cells, cathepsin D, a prototype lysosomal enzyme, is synthesized as a 51-kDa polypeptide with two *N*-linked oligosaccharides co-translationally attached. After transport to the Golgi complex, the oligosaccharide chains are modified and mannose residues are phosphorylated (11). This results in the appearance of a heterogeneous population of cathepsin D species with apparent molecular masses of 51–55 kDa as determined by SDS-PAGE. Following binding to mannose phosphate receptors (MP-R), procathepsin D is transported to endosomes, where it is proteolytically processed to a 44-kDa polypeptide. Subsequently, it arrives in the lysosomes and is converted into the (31-kDa) mature enzyme (24). First, we determined the time required for transport from the site of synthesis (rough ER) to the medial Golgi complex using the fact that a small percentage of the *N*-linked oligosaccharides of cathepsin D becomes resistant to Endo H. Cathepsin D was labeled during a 15-min pulse with [^{35}S]methionine. The front of labeled cathepsin D reached the medial Golgi after 30 min, as can be concluded from the appearance of Endo H-resistant cathepsin D (Fig. 5*A*). This is consistent with the transport rate reported for fibroblasts (1). Fig. 5*A* also shows that the front of cathepsin D (indicated by the appearance of the 31-kDa species) reached the lysosomes after 75 min of chase.

To determine the effect of BFA on the processing and transport of cathepsin D, HepG2 cells were pulse-labeled with [^{35}S]methionine and then chased for 4 h. At different time points during the chase, BFA was added. Since the effect of

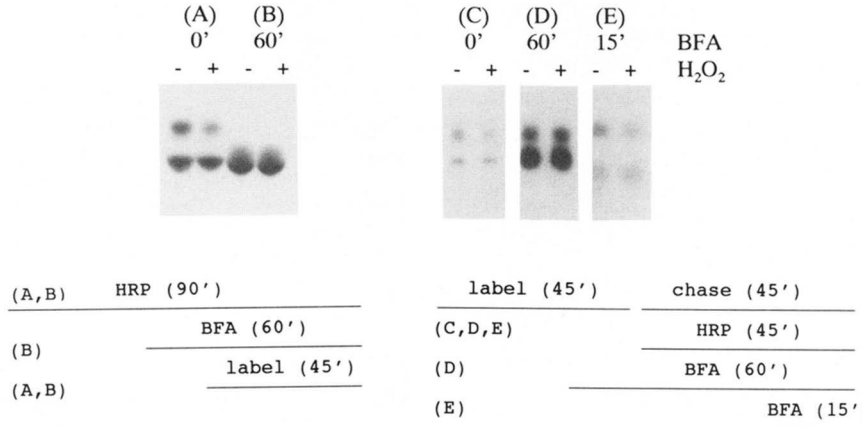

FIG. 4. **Accessibility of α_1-antitrypsin-containing compartments for endocytosed horseradish peroxidase (*HRP*).** Cells (6-cm plates) were incubated in MEM containing 3 mg/ml horseradish peroxidase. After 30 min of uptake, BFA was added (*B*), and after another 15 min, the cells were labeled for 45 min with [^{35}S]methionine (*A* and *B*) in BFA (*B*) and horseradish peroxidase (*A* and *B*)-containing medium. In *C–E*, cells were labeled with [^{35}S]methionine for 45 min and chased in the presence of 3 mg/ml horseradish peroxidase for another 45 min. In *D* and *E*, BFA was added 15 min before and 30 min after the start of the chase period, respectively. The cells were then released from the dish by treatment with proteinase K at 0 °C, and DAB cytochemistry was performed. After lysing the cells, α_1-antitrypsin was immunoprecipitated and analyzed by SDS-PAGE. The loss of complex signal by the DAB cytochemistry, determined in three independent experiments, was expressed as percentage of the control, normalized to the loss of the high mannose form.

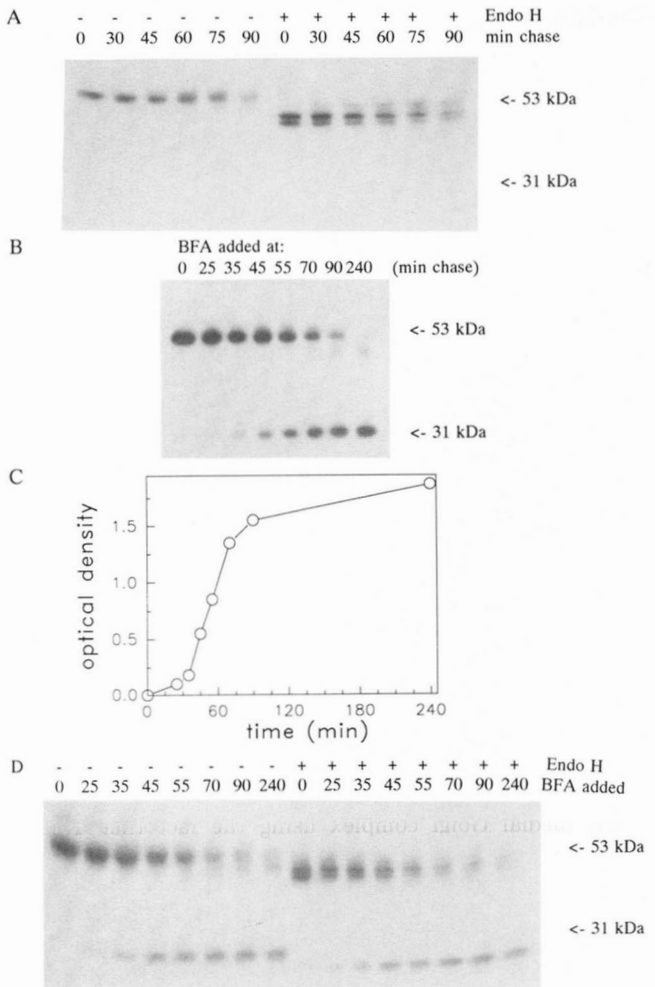

FIG. 5. **Effect of BFA on transport of cathepsin D.** *A*, cells (35-mm plates) were labeled for 15 min with [^{35}S]methionine and chased in the presence of excess unlabeled methionine for the periods of time indicated. Next, the cells were lysed, and cathepsin was quantitatively immunoprecipitated. The immunoprecipitates were split in two equal portions, incubated with Endo H (+) or without Endo H (−) and analyzed by SDS-PAGE. *B*, cells were labeled as in *A* and chased for 240 min. At the indicated times, BFA (10 μg/ml) was added to the chase medium. The cells were lysed, and cathepsin D was immunoprecipitated and analyzed by SDS-PAGE. In *C*, the relative amounts of 31-kDa cathepsin D is plotted after scanning the fluorograph in *B*. *D*, experiment was as in *B*; the immunoprecipitates were split in two equal portions, incubated with (+) or without (−) Endo H, and analyzed by SDS-PAGE.

BFA is instantaneous (as determined morphologically and biochemically), this experiment allows a determination of the site at which BFA affects transport (Fig. 5, *B* and *C*). The quantitation of the appearance of 31-kDa cathepsin D is shown in Fig. 5*C* and demonstrates that BFA exerts its effect during a sharp interval between 45 and 70 min after the beginning of the chase. Addition of BFA at the beginning of the chase period (after 15 min of pulse) prevented maturation of cathepsin completely. If BFA was added 55 min after the start of the chase, 50% of ^{35}S-labeled cathepsin D was processed to the mature 31-kDa cathepsin D, whereas virtually complete conversion was obtained if no BFA was added (240 min). Only a little cathepsin D was not converted to the mature form if BFA was added after 90 min of chase. Since 31-kDa cathepsin D is formed in lysosomes (24), processing to the mature form is an indication of transport to lysosomes. Confirmation that the 31-kDa cathepsin D, processed in the presence of BFA, was indeed transported to lysosomes was obtained by buoyant density fractionation on Percoll gradients. The 31-kDa species was recovered in the same fractions as the lysosomal enzyme activity β-hexosaminidase (Fig. 6). In this case, the cells were chased for 3 h. As seen in *panel B*, the 51–53-kDa procathepsin D remains in light density fractions, whereas the 44-kDa species is completely converted to the 31-kDa form.

BFA exerts its effect on the transport of cathepsin D either at the transport step from ER to Golgi only or both at the transport from ER to Golgi and between TGN and endosome. To discriminate between these possibilities, we repeated the experiment of Fig. 5*B* and used Endo H to answer the question of whether BFA addition at various time points during a 240-min chase causes accumulation of Endo H-resistant procathepsin D (Fig. 5*D*). No accumulation of Endo H-resistant 53-kDa procathepsin D was detectable. This experiment shows that once cathepsin D has passed the medial Golgi complex (*i.e.* the site at which it becomes partially resistant to endo H) it is transported further to lysosomes independent of the presence of BFA.

If transport mediated by MP-R from the Golgi complex to endosomes is not affected by BFA, MP-R-mediated endocytosis (uptake) of cathepsin D may continue as well. To test this, [^{35}S]methionine-labeled procathepsin D, synthesized and secreted by cells grown in the presence of 10 mM NH_4Cl, was collected and, after dialysis, added to unlabeled HepG2 cells (Fig. 7). Uptake was mediated exclusively by MP-R, since addition of 10 mM mannose 6-phosphate to the uptake medium reduced uptake of [^{35}S]methionine-labeled cathepsin D almost completely (Fig. 7, *lane 3*). After 4 h of continuous uptake, most of the endocytosed procathepsin D was efficiently processed to the 44-kDa intermediate and to the 31-kDa mature forms (Fig. 7, *lane 1*). In the presence or absence

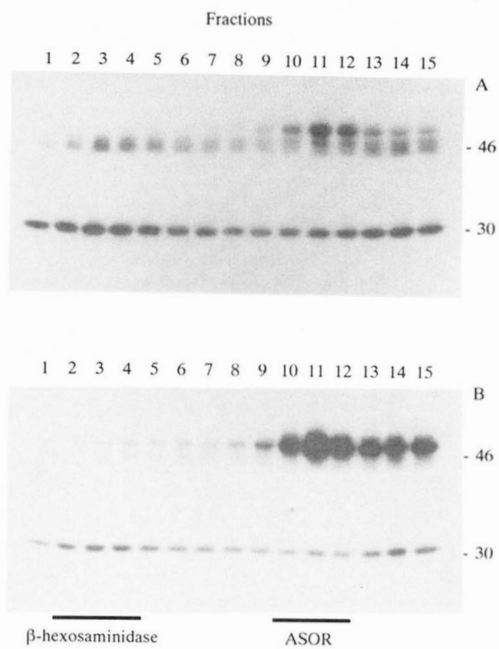

FIG. 6. **Effect of BFA on the subcellular distribution of cathepsin D-containing vesicles on Percoll density gradients.** HepG2 cells (6-cm plates) were labeled with [^{35}S]methionine for 75 min to fill the biosynthetic pathway with labeled cathepsin D species. Then the cells were chased for 3 h in the absence (*A*) or presence of 5 μg/ml BFA (*B*). The presence of lysosomes was determined by β-hexosaminidase enzyme assay, and the light density vesicles (containing endosomes, ER, and Golgi in this Percoll gradient) were identified by the uptake of ^{125}I-labeled asialo-orosomucoid (*ASOR*) after a short uptake period (10 min).

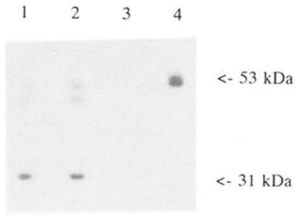

FIG. 7. **Effect of BFA on endocytosis and processing of cathepsin D.** HepG2 cells (6-cm dish) were labeled for 4 h with [^{35}S] methionine in the presence of 10 mM NH_4Cl. NH_4Cl was removed by dialysis against MEM. ^{35}S-Labeled procathepsin D was immunoprecipitated from the release medium and analyzed by SDS-PAGE (*lane 4*). Unlabeled cells (35-mm plates) were incubated with equal amounts of the [^{35}S]methionine-labeled total secreted protein for 4 h in the absence of BFA (*lane 1*), in the presence of 10 μg/ml BFA (*lane 2*), or in the presence of 10 mM mannose 6-phosphate (*lane 3*). Cathepsin D was immunoprecipitated and analyzed by SDS-PAGE.

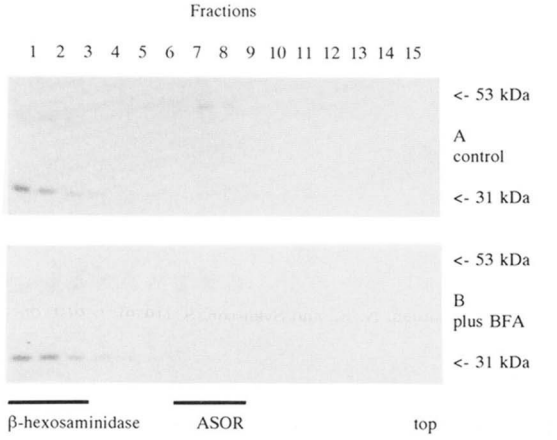

FIG. 8. **Subcellular fractionation of cathepsin D-containing vesicles on Percoll density gradients.** Unlabeled HepG2 cells were incubated in the presence of [^{35}S]methionine-labeled total secretory protein in the presence (*lower panel*) or the absence of BFA (*upper panel*) as described in the legend to Fig. 7. The cells were then homogenized, and the postnuclear supernatants were fractionated on Percoll gradients. Subsequently, cathepsin D was immunoprecipitated from the fractions and analyzed by SDS-PAGE. The three bottom fractions contained the lysosomes as was monitored by the presence of β-hexosaminidase. The fractions in the middle of the gradients contain smooth membranes, including endosomes, Golgi, and most of the rough endoplasmic reticulum. The presence of endosomes was shown by uptake of ^{125}I-labeled asialo-orosomucoid for 5 min (*ASOR*). The same fractions contain the galactosyltransferase enzyme activity.

of BFA, roughly equal amounts of the mature cathepsin D were recovered from the cell lysates. The only effect of BFA was on the relative amounts of 53- and 44-kDa intermediates; in the presence of BFA, more labeled precursors were present. This is probably due to a redistribution of MP-R in the presence of BFA resulting in higher numbers of receptors at the cell surface (3, 35). To ascertain that the transport of internalized cathepsin D occurred normally, ^{35}S-labeled cathepsin D-labeled cells were fractionated on Percoll density gradients (Fig. 8). The distribution of ^{35}S-labeled cathepsin D precursors, as well as mature ^{35}S-labeled cathepsin D was independent of the presence of BFA. These experiments clearly show that, once cathepsin D is directed to endosomes, further transport to lysosomes and proteolytic processing are unaffected by BFA.

DISCUSSION

In a number of cell lines, protein traffic from ER to cis-Golgi is inhibited by BFA, resulting in a net backward transport of Golgi constituents to the ER (15, 16, 33). Our study shows that HepG2 cells respond to BFA in a similar manner as was monitored by a redistribution of UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase, sialyltransferase, and UDP-glucose:ceramide glucosyltransferase to the ER (Fig. 1). In addition, BFA induced a transport block for the secretory protein α_1-antitrypsin at the TGN (Figs. 2–4). In contrast, transport of the lysosomal hydrolase cathepsin D from TGN to the degradative pathway was not inhibited upon BFA treatment (Figs. 5 and 6). BFA also did not interfere with the processing of procathepsin D and its targeting to lysosomes after MP-R-mediated endocytosis (Figs. 7 and 8).

Addition of BFA to the cell culture media causes a very rapid but reversible rearrangement of Golgi elements (5, 7, 15, 20). Resident membrane proteins of the cis-, medial, and trans-Golgi, but not of the TGN, are rapidly redistributed to the rough ER (15, 17, 35). The extent of these redistributions has not been quantitated precisely, except for UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase, which we reported to be partially (70%) shifted to the rough ER (32). In the present study, we have confirmed and extended these observations to a second trans-Golgi enzyme, sialyltransferase, and an enzyme thought to be located in the cis-Golgi, UDP-glucose:ceramide glucosyltransferase (8, 13). The observation that 70% of the sialyltransferase was recovered in vesicles that cofractionated with the ER confirms the notion that in HepG2 cells trans-Golgi constituents are also transferred to the ER. It seems likely that the fraction of the enzymes that remained at a Golgi location on gradients actually resided in the TGN. The nearly complete shift of UDP-glucose:ceramide glucosyltransferase to ER upon BFA treatment is in accordance with its proposed location in cis-Golgi. Although it cofractionates with trans-Golgi markers (13), Futerman *et al.* (8) have provided arguments for the location of part of the UDP-glucose:ceramide glucosyltransferase activity in the intermediate compartment.

The secretory protein α_1-antitrypsin was used as a prototype of secretory proteins to show the effect of BFA on protein transport through the various Golgi subcompartments. As expected in the presence of BFA, high mannose α_1-antitrypsin remained in the rough ER or was transported back from the cis-Golgi to the rough ER in the presence of BFA. However, most of the complex α_1-antitrypsin remained intracellularly and was *not* transported back to the rough ER. Assuming that constitutive secretory proteins can freely diffuse within the organelles and are transported as bulk phase after the rough ER to the cell surface in hepatoma cells (18), we conclude that BFA induced a second transport block in the secretory pathway. Since this block occurred in a compartment containing complex glycosylated secretory protein that was recovered in sucrose gradients in Golgi-containing fractions, we conclude that this compartment would either be the transcisterna of the Golgi stack or the TGN. The former possibility is unlikely because true marker enzymes of this compartment such as UDP-galactose β-D-*N*-acetylglucosamine β1–4-transferase and sialyltransferase are mainly redistributed to ER-containing fractions upon BFA treatment, whereas complex-type α_1-antitrypsin remained almost completely in low density vesicles. Furthermore, the compartment contained endocytosed horseradish peroxidase (and transferrin-horseradish peroxidase)[2] when added to the cells before BFA. This strongly suggests that this compartment is derived from the TGN, since we have demonstrated that transferrin-horseradish peroxidase endocytosed by the transferrin receptor (29)

[2] G. J. Strous, P. van Kerkhof, G. van Meer, S. Rijnboutt, and W. Stoorvogel, unpublished observations.

and fluid phase-endocytosed horseradish peroxidase (24) label the site in the secretory pathway at the TGN. It is also consistent with the recent findings of Reaves and Banting (23), who observed that the TGN collapses around the microtubule-organizing center in the presence of BFA.

It has been reported for various cell types that TGN markers or proteins that had reached the TGN via endocytosis were not redistributed to the ER in the presence of BFA (2, 15, 17, 23, 26, 35). The present work confirms both notions for HepG2 cells. No significant amounts of complex α_1-antitrypsin were transported back to the ER. Neither did BFA induce detectable transport to the ER of fluid phase-endocytosed horseradish peroxidase or transferrin receptor-bound transferrin-horseradish peroxidase[2] that had been accumulated in endosomes and TGN prior to BFA addition.

In the presence of BFA the receptors for mannose phosphate-containing ligands and transferrin continue their recycling between the plasma membrane and endosomes (3, 17). Our data on the uptake of mannose phosphate-linked cathepsin D are in agreement with this (Fig. 7). It has been suggested, based on the assumption that TGN-38 is a specific marker for the TGN, that BFA induces the formation of a continuous network between TGN and endosomes (17, 35). Our data show that both biosynthetic targeting beyond the trans-Golgi and endocytic targeting of cathepsin D continues in the presence of BFA. A continuous network of TGN and endosomes might therefore be functioning in targeting cathepsin D to lysosomes via both pathways. Thus, mannose phosphate-containing proteins from both the biosynthetic route and from outside the cell can probably be transported in endosomes in the presence of BFA. In endosomes, lysosomal enzymes detach from their receptors (MP-R) and continue to the lysosomes as fluid phase constituents (14). This process is not fully understood. Based on the effect of BFA on uptake and transport of fluid phase-endocytosed markers, Lippincott-Schwartz *et al.* (17) assume that endosomes and lysosomes are different homotypic membrane systems, between which transport is inhibited in the presence of BFA. Our experiments are not consistent with these observations and demonstrate that transport of cathepsin D from endosomes to lysosomes is unaffected by BFA. In conclusion, our data show that BFA exerts a differential effect on the transport of a secretory and a lysosomal protein; secretion of α_1-antitrypsin is inhibited at the TGN, whereas both transport from TGN to lysosomes as well as endocytic transport of cathepsin D continues.

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