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Brefeldin A (BFA) Inhibits Basolateral Membrane (BLM) Delivery and Dimerization of Transcobalamin II Receptor in Human Intestinal Epithelial Caco-2 Cells

BFA EFFECTS ON BLM CHOLESTEROL CONTENT*

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Brefeldin A (BFA) treatment of Caco-2 cells (5 μg/ml for 12 h) reduced by 90% the cholesterol, but not the phospholipid (PL), levels of the basolateral membrane (BLM), thus altering its PL/cholesterol molar ratio from 2.6 to 22.0, and decreasing its steady state fluorescent anisotropy (r_s) from 0.27 to 0.15. BFA treatment for 12 h also resulted in complete loss of transcobalamin II receptor (TC II-R) activity/protein levels in the BLM and the disappearance of trans-Golgi network (TGN) morphology as revealed by confocal immunofluorescence microscopy using antibody to TGN 38. However, BFA treatment had no effect on either total cellular cholesterol, TC II-R activity, or PL levels. When cells treated with BFA for 12 h were exposed to BFA-free medium for 0–24 h, all of the effects were reversed, including reappearance of normal TGN morphology. TC II-R delivered to the BLM during this period was progressively sialylated and changed its physical state from a monomer (8 h) to a dimer (12 h), coinciding with increased delivery (11–53 pmol) of cholesterol to the BLM and an increase in the BLM r_s from 0.15 to 0.21. These results indicate that cholesterol, but not PL, delivery to the BLM of Caco-2 cells is BFA-sensitive, and cholesterol, by influencing the higher order of the BLM, is essential for TC II-R dimerization.

Circulatory cobalamin (Cbl[1]; vitamin B_{12}) bound to plasma transporter, transcobalamin II (TC II), is taken up by all tissues/cells by receptor-mediated endocytosis via plasma membrane (PM) transcobalamin II receptor (TC II-R) (1). TC II/TC II-R-mediated delivery of Cbl is the only physiological uptake system that provides Cbl to all cells to be utilized as Cbl coenzyme. TC II-R, a glycoprotein with a molecular mass of 62 kDa (2) is expressed in all tissue PMs as a noncovalent functional homodimer with a molecular mass of 124 kDa (2). TC II-R homodimers are resistant to treatment with sodium dodecylsulfate (2, 3) and, thus, can be separated on SDS-PAGE and detected by immunoblotting (3). Studies using this technique (4) have revealed that at steady state, TC II-R dimer levels are 8–10-fold higher than that of the monomer in all total tissue membranes tested and that TC II-R dimers are present in the PM and in some PM-derived vesicles, while TC II-R monomers are the only species present in the microsomes (4).

Earlier *in vitro* studies (3) using isolated tissue PMs and microsomes have revealed that TC II-R dimerization is supported in the plasma but not in the microsomal membranes due to their higher cholesterol content. Additional studies using symmetrical phosphatidylcholine (PC) vesicles have shown that a minimum of 10 mol % of cholesterol is essential to support TC II-R dimerization above the transition temperatures of these PC vesicles (3). Although the importance of membrane cholesterol levels and cholesterol-phospholipid interactions in the dimerization of TC II-R in tissue-derived PMs and in PC vesicles is established (3), it is not known how the dimerization of TC II-R is regulated at a cellular level and whether cellular PM cholesterol levels are important for the dimerization of TC II-R.

These issues have been addressed in the current study using polarized epithelial Caco-2 cells that express TC II-R predominantly (85%) in the BLM (5, 6) and are known to be sensitive to treatment with BFA (7). BFA, a fungal metabolite, causes Golgi disruption and other morphological changes (8–13) in cells and has been widely used to study the effect of these changes on the sorting of proteins (14, 15) and lipids (16, 17) in a variety of cells. Our aim in the current study was to test whether BFA affected BLM delivery of major lipids such as cholesterol and PL in Caco-2 cells and, if so, whether it also affected the BLM delivery and dimerization of TC II-R.

The results of the current study show that in Caco-2 cells, delivery of cholesterol, but not PL, to the BLM is BFA-sensitive. As a consequence of decreased cholesterol levels, TC II-R monomers delivered to the BLM during the incubation of BFA-treated cells with BFA-free medium were able to dimerize only after 12 h of incubation, when cholesterol levels of the BLM and its order were restored to nearly normal values.

EXPERIMENTAL PROCEDURES

Materials—[^{57}Co]Cyanocobalamin (specific activity 15 μCi/μg) was from Johnson and Johnson Clinical Diagnostics (Ontario, Canada),

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[1] The abbreviations used are: Cbl, cobalamin (vitamin B_{12}); DMEM, Dulbecco's modified Eagle's medium; TC II-R, transcobalamin II receptor; TC II, transcobalamin II; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; PM, plasma membrane; PL, phospholipid; PC, phosphatidylcholine; r_s, steady state fluorescence anisotropy; TGN, trans-Golgi network; TBS, Tris-buffered saline; BLM, basolateral membrane; BFA, brefeldin A.

carrier-free Na^{125}I was from Amersham Pharmacia Biotech, and human serum was obtained from Southeastern Wisconsin Blood Center (Milwaukee, WI). Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were from Life Technologies, Inc.; [^{35}S]methionine (>1000 Ci/mmol) was from NEN Life Science Products; protein A, BFA, lactoperoxidase, and neuraminidase from *Clostridium perifringens* were from Sigma; and endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus*, peptide:*N*-glycosidase F from *Flavobacterium meningosepticum,* and *O*-glycosidase from *Diplococcus pneumonia* were from Boehringer Mannheim. Disuccinimidyl suberate sulfosuccinimido biotin and IODO-GEN were from Pierce. 1-(4-Trimethylamino)-phenyl-6–1,3,5-triene was from Molecular Probes, Inc. (Eugene, OR), and purified *Sanbiscus nigra* agglutinin covalently linked to agarose beads was from E-Y Laboratories (San Mateo, CA). Millicell-HA culture plate inserts were from Millipore Corp., and Nitrocellulose membranes were from Schleicher and Schuell. Pure human TC II was a gift from the late Dr. Charles A. Hall (Nutrition Assessment Laboratories, Veterans Affairs hospital, Albany, NY). Antiserum to human TC II-R was prepared in rabbits as described earlier (2). Rabbit anti-rat polyclonal antibody against TGN 38 was a gift from Dr. Andrew R. Wilde (Department of Pharmacology, University of California, San Francisco). The secondary antibody was purchased from Jackson ImmuneResearch Laboratories (West Grove, PA).

Cell Culture—Caco-2 cells (passages 76–80) were routinely grown in DMEM (25 mM glucose) as described earlier (5, 6). For ligand binding and domain-specific biotinylation studies cells were grown as epithelial layers by high density seeding (1.5×10^6 cells/filter) onto membrane filter inserts (Millicell-HA, 30-mm diameter, 0.45-μm pore size) and used as described before (5, 6).

Digestion of ^{35}S-TC II-R with Glycosidases—Postconfluent Caco-2 cells grown on plastic using 75-cm^2 flasks were first incubated in the presence and absence of BFA (5 μg/ml) for 12 h at 37 °C. The medium was replaced with methionine-free DMEM, and the cells were incubated for 30 min. The cells were then labeled with [^{35}S]methionine (200 μCi/flask/8 ml of medium) for 60 min. BFA (5 μg/ml) was present during both these incubations. The cells were then harvested, washed, and homogenized in 1 ml of Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.1 mM phenylmethylsulfonyl fluoride (TBS). The homogenate was then treated with Triton X-100 (1%), and the detergent extract was immunoprecipitated with TC II-R antiserum (25 μl) and protein A-Sepharose (125 μl of 1:1 suspension). The radioactive counts were liberated by boiling with SDS (1%), and the liberated radioactivity was subjected to acetone precipitation to remove SDS. The precipitated radioactivity was further processed for treatment with various glycosidases essentially as described earlier (18).

Binding of ^{35}S-TC II-R to S. nigra Agglutinin-Agarose—Postconfluent Caco-2 cells that were incubated for 12 h in the absence or presence of BFA (5 μg/ml) were pulsed for 1 h with [^{35}S]methionine (200 μCi/flask). In some experiments, the cells treated with BFA were incubated with BFA free medium for 0–16 h. The cells were then extracted with TBS containing Triton X-100 and centrifuged. The extract was immunoprecipitated with TC II-R antiserum (25 μl) and protein A-Sepharose (125 μl). The radioactivity immunoprecipitated (25,000–28,000 cpm) was liberated with TBS containing SDS (1%) followed by precipitation with acetone to remove SDS. The precipitated radioactivity was resolubilized with TBS containing Triton X-100 (1%), and the solubilized radioactivity (20,000 cpm) was incubated with *S. nigra* agglutinin-agarose beads. The beads were washed with TBS, and the beads were pelleted and counted. As a control, ^{35}S-TC II-R immunoprecipitated (20,000 cpm) from cells not treated with BFA was digested with sialidase and then allowed to bind to *S. nigra* agglutinin-agarose beads, and the radioactivity bound was washed and counted as before. The radioactivity eluted from *S. nigra* agglutinin-agarose beads was subjected to nonreducing SDS-PAGE.

Iodination of TC II and Streptavidin—Human TC II (5 μg) and streptavidin (50 μg) were each iodinated with 0.5 mCi of Na^{125}I and IODO-GEN, as recommended by the manufacturer (Pierce). The specific activity of iodinated TC II and streptavidin was 70–75 μCi/μg and 6–7 μCi/μg, respectively.

Cross-linking of BLM with ^{125}I-TC II-Cbl—Filter grown Caco-2 cells that were exposed to BFA (5 μg/ml) for 12 h were washed and then incubated with BFA-free DMEM for 8–24 h. The medium was removed, and the filters were incubated in the presence and absence of TC II-Cbl (10 pmol) on the basolateral side with DMEM containing ^{125}I-TC II-Cbl (500 fmol, 200,000 dpm) for 30 min at 4 °C. The cells were washed with fresh medium and then incubated with 4 mM disuccinimidyl suberate for 30 min at 4 °C. The cross-linking reaction was stopped by the addition of glycine (0.1 mM). The cells were harvested and homogenized (1 ml of TBS), and the total membrane collected by centrifugation was then analyzed on nonreducing SDS-PAGE (5%). The bands were visualized after autoradiography.

SDS-PAGE and Immunoblotting—Isolated total membranes (25 μg of protein) from Caco-2 cells that were exposed to BFA (0, 2, 5, 10 μg/ml) for 12 h or cells that were allowed to recover from BFA effects for various times (0–24 h) were subjected to nonreducing SDS-PAGE (7.5%) (19). Separated proteins were electroblotted for either 45 or 90 min at 90 V to detect optimal levels of TC II-R monomer and dimer forms, respectively, onto nitrocellulose membranes and probed with TC II-R antiserum and ^{125}I-protein A as described previously (3). Immunoblots (see Figs. 2, 3, and 7) were repeated at least three times using fractions from three separate experiments, and some immunoblots were quantified by the AMBIS radioimaging system as described previously (4).

Cell Surface Biotinylation—Biotinylation of BLM of Caco-2 cells recovering from the effects of BFA was carried out by adding disuccinimidyl suberate sulfosuccinimido biotin (0.5 mg/ml) to the basolateral compartments of filter-grown monolayers (12-day growth) and was performed a total of three times for 30 min each essentially as described recently (6, 18).

Anisotropy Measurements—Postconfluent Caco-2 cells grown on culture inserts were incubated with BFA (5 μg/ml) for 12 h. The medium was replaced with fresh medium without BFA, and the cells were incubated for 0–24 h. The cationic probe trimethylammoniumdiphenyl hexatriene dissolved in dimethyl sulfoxide (0.1 pmol/filter) was added to the basolateral medium 30 min before the allocated time of 2–24 h. The cells were then harvested at each time interval, washed in TBS, and finally suspended in 3 ml of TBS and used for fluorescent polarization studies in a 3-ml quartz cuvette with constant stirring (250 rpm). Fluorescence anisotropy (r_s) was determined at room temperature using a model 4800 C spectra fluorometer (SLM-Aminco Inc., Rochester, NY). The excitation and emission wavelengths were 360 and 430 nm, respectively. Correction for light scattering was carried out by successive dilutions of the cell suspension until a plateau value of polarization was obtained. Steady state r_s was determined at least 10 times for each sample at each dilution from three sets of cells treated with trimethylammoniumdiphenyl hexatriene from three separate experiments, and the r_s values were calculated according to Van Blitterswijk *et al.* (20).

Pulse-Chase Labeling of Caco-2 Cells—Postconfluent cells untreated and treated with BFA (5 μg/ml) for 12 h were first incubated with methionine-free DMEM for 30 min and then pulsed for 1 h with [^{35}S]methionine (200 μCi/flask) in the presence of BFA. The medium was removed, and the cells were washed with DMEM and then chased for 0–16 h with DMEM containing methionine (20 mM and BFA). The ^{35}S-labeled TC II-R isolated at each time interval by immunoprecipitation was further processed for nonreducing SDS-PAGE as described before (18).

Confocal Immunofluorescence Microscopy—Postconfluent Caco-2 cells grown on filters were incubated in the presence and absence of BFA (5 μg/ml) for 12 h. In some experiments, the cells were allowed to recover from the effects of BFA by incubation in BFA-free medium for 24 h. Following incubations, the filters were washed with phosphate-buffered saline and fixed in 4% paraformaldehyde followed by sequential incubations with the primary antibody (rabbit polyclonal TGN 38 antibody) for 1 h at 37 °C and Texas red-conjugated secondary antibodies (donkey anti-rabbit) for 30 min at 37 °C in the dark. The samples were then analyzed using a krypton-argon laser coupled with a Bio-Rad MRC 600 confocal head attached to an Optiphot II Nikon microscope with a plan Apo 60 × 1.4 NA objective lens.

Other Methods—Reconstitution of pure sialylated and asialo-TC II-R in total BBM lipid extracts was carried out using 2 μmol of total PL and either sialylated or asialo-TC II-R (0.25 μg) as described previously (3). Protein concentration was determined using the Bradford assay with bovine serum albumin as the standard (21). TC II-R assays were performed using partially purified TC II from human serum (22). TC II-[^{57}Co]Cbl complex was prepared for receptor assays by the charcoal adsorption method (23). Basolateral cell surface binding of human TC II-[^{57}Co]Cbl (500 fmol) using filter-grown Caco-2 cells was determined by incubating the ligand at 4 °C for 30 min. After 30 min, the medium was removed, cells were washed in cold medium, and the amount of TC II-[^{57}Co]Cbl bound to the surface membrane was determined by counting the radioactivity in scraped cells. The TC II-R-specific ligand binding was then calculated by subtracting the amount of ligand bound to the cell surfaces in the presence of TC II-R antiserum (5–20 μl) or that bound when the ligand was incubated at 5 °C in the presence of pH5/EDTA buffer. In general, the nonspecific binding was less than 5% of the total ligand bound. Total membranes from scraped Caco-2 cells

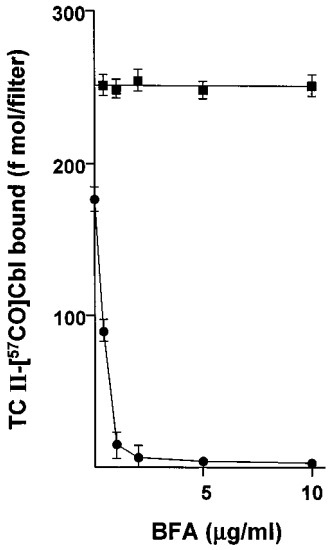

FIG. 1. **Effect of different concentrations of BFA on the total and BLM TC II-R activity.** Caco-2 cells grown on Millipore-HA filters were incubated on both the apical and the basolateral side with the indicated concentrations of BFA for 12 h at 37 °C. In some filters, ligand binding to the BLM ( ) was determined by the addition of TC II-[^{57}Co]Cbl(500 fmol). The filters were incubated for 1 h at 4 °C. The cells were then scraped, and the radioactivity was counted. Specific binding was determined by subtracting total radioactivity bound from the radioactivity bound to BLM in the presence of TC II-R antiserum (25 μl). Total cellular TC II-R activity (■) was determined as described earlier (2) using Triton X-100 extracts of the cell homogenates. Other details are provided under "Experimental Procedures." Each data point represents mean ± S.D. of triplicate assays from three separate filter experiments.

were prepared by collecting the pellet obtained by centrifuging a 10% homogenate of the cells prepared in TBS containing 0.25 M sucrose, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride at 100,000 × *g* for 60 min. BLMs from BFA-treated and -untreated cells were isolated according to Ellis *et al.* (24). The BLM fraction was enriched for TC II-R, and a marker enzyme, Na^+/K^+ ATPase, was enriched 15- and 17-fold with 18 and 20% recoveries, respectively. Total lipid extract was prepared from total cellular homogenate (2 mg of protein) and the isolated BLM (210–220 μg of protein) by extraction with chloroform/methanol (2:1). The organic layer was removed and used for total PL and cholesterol estimation as described previously (3).

RESULTS

BFA Inhibits BLM but Not the Total Cellular TC II-R Activity or Protein Levels in Filter-grown Caco-2 Cells—Incubation of Caco-2 cells for 12 h with BFA (0.5–10 μg/ml) revealed inhibition of BLM but not total cellular ligand binding by 50 and 100% at BFA concentrations of 0.5 and 5 μg/ml, respectively (Fig. 1). When incubated with 5 μg/ml BFA for less than 12 h, ligand binding to BLM was inhibited by 50, 75, and 90% inhibition occurring at 2, 4, and 8 h of incubation, respectively. Thus, in all subsequent experiments cells were incubated with 5 μg/ml BFA for 12 h.

Immunoblotting of total cell membranes (Fig. 2) from untreated cells revealed that TC II-R was a dimer with a molecular mass of 124 kDa (Fig. 2, *left panel*), and TC II-R monomer with a molecular mass of 62 kDa could not be detected (Fig. 2, *right panel*). When cells were incubated with 2, 5, and 10 μg/ml BFA for 12 h, concentrations that inhibited BLM TC II-R activity by >95%, the dimer form of TC II-R disappeared (Fig. 2, *left panel*), and the monomer form was the only form present (Fig. 2, *right panel*). Image density quantitation (data not shown) of these bands revealed equal intensity, suggesting that there was no loss of TC II-R protein. However, the size of TC II-R monomer was 56 kDa, nearly 6 kDa less than the size of monomeric TC II-R in untreated cells. Pulse-chase labeling of

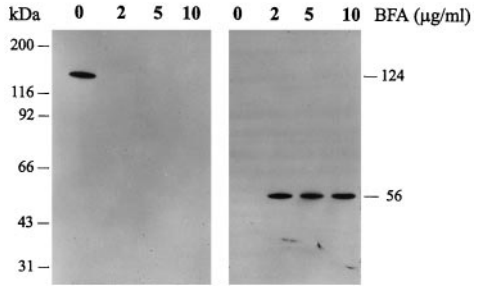

FIG. 2. **Immunoblot analysis of total membranes from Caco-2 cells incubated with different concentrations of BFA.** Caco-2 cell total membranes (25 μg of protein) isolated from Caco-2 cells that were incubated for 12 h at 37 °C with indicated concentrations of BFA were subjected to nonreducing SDS-PAGE (7.5%). The proteins separated were transferred for either 90 (*left panel*) or 45 min (*right panel*) to nitrocellulose, probed with TC II-R antiserum and ^{125}I-protein A, and visualized as described before (3).

Caco-2 cells in the presence and absence of BFA (5 μg/ml) revealed on SDS-PAGE a single immunoprecipitated band of 56 and 62 kDa, respectively, and the $t_{1/2}$ of TC II-R in both cases was 7.5 h (data not shown). The pulse-chase experiment supported further the findings of the immunoblot using total membranes (Fig. 2) that BFA treatment did not alter the turnover of TC II-R and that the size of intracellularly retained TC II-R monomer was lower by 6 kDa.

Immunoblotting of total membranes of Caco-2 cells recovering from the effects of BFA (Fig. 3) revealed TC II-R dimers with a molecular mass of 124 kDa in untreated cells (*left panel*, *lane C*) and from cells that had recovered from the effects of BFA for 12 and 24 h, but not for 0–8 h. TC II-R monomer was detected only in cells that had recovered from BFA treatment for 0–8 h (Fig. 3, *right panel*) and not in untreated cells (*right panel*, *lane C*). Since TC II-R dimers were detected in the total membranes only after 12 h of recovery from BFA, we wanted to test during the different recovery periods whether TC II-R is delivered to the BLM and, if so, whether it is delivered as a monomer or as a dimer.

Inhibition of Basolateral TC II-R Activity/Protein Is Reversible and TC II-R Is Delivered to the BLM as a Monomer—When the filter-grown cells exposed to BFA for 12 h were incubated with BFA-free medium for 2–24 h, there was a gradual recovery of BLM ligand binding activity (Fig. 4, *left panel*) with 50 and 95% recovery at 8 and 16 h, respectively. Biotinylation of BLM followed by SDS-PAGE of the immunoprecipitated extract of cells during the recovery period (Fig. 4, *right panel*) revealed progressive recovery of TC II-R protein with time (0–24 h), and the molecular mass of TC II-R increased from 56 to 62 kDa during this period. Quantitation of these bands (Fig. 4, *left panel*) revealed a linear relationship between the recovery of TC II-R protein and the BLM ligand binding activity. Nearly 50% recovery of ligand binding and TC II-R protein levels occurred following 8 h of exposure to BFA-free medium when the molecular mass of TC II-R was less than 62 kDa.

TC II-R detected on SDS-PAGE (Fig. 4, *right panel*) used samples that were extracted with Triton X-100, a treatment that completely solubilizes TC II-R and converts it to the monomeric form (2, 3). Thus, it was not possible to assess whether TC II-R delivered to BLM during the recovery was a monomer or a dimer. However, chemical cross-linking the BLM of intact cells grown on culture inserts with ^{125}I-TC II-Cbl (Fig. 5) during the recovery periods revealed a cross-linked product with a molecular mass of 99–100 kDa (*lane 2*) at 8 h of reversal time, while it was 210 kDa at 12 (*lane 3*) and 24 (*lane 4*) h of reversal time. The size of 99 kDa of the receptor-ligand complex for BLM at 8 h of reversal is consistent with the binding of the 43-kDa

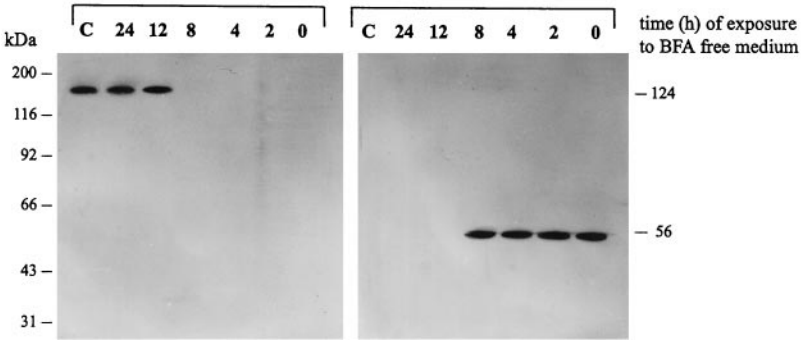

FIG. 3. **Immunoblot analysis of total membranes from Caco-2 cells incubated for 12 h with BFA and then in BFA-free medium-Caco**. Two cell total membranes (25 μg protein) isolated from Caco-2 cells that were first incubated with BFA (5 μg/ml) for 12 h at 37 °C and later incubated in the absence of BFA for indicated time intervals were subjected to nonreducing SDS-PAGE (7.5%) and immunoblotting as before.

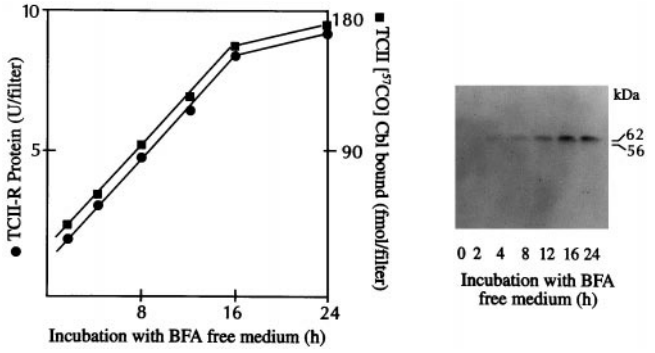

FIG. 4. **Biotinylation of BLM of filter-grown Caco-2 cells**. Postconfluent Caco-2 cells grown on Millipore culture inserts were first incubated with BFA (5 μg/ml) for 12 h and later in BFA-free medium for the indicated time intervals. At each indicated time, the BLMs of some filters were biotinylated with disuccinimidyl suberate sulfosuccinimido biotin as described under "Experimental Procedures," while others were used for binding to the ligand. Ligand binding to BLM (*left panel*) was carried out using TC II-[^{57}Co]Cbl (500 fmol) as described before (6). The biotinylated cells were scraped, extracted with Triton X-100(1%), immunoprecipitated with antiserum to TC II-R, and subjected to nonreducing SDS-PAGE (7.5%), and the bands (*right panel*) were detected by probing with ^{125}I-streptavidin as described before (6). The bands shown in the *right panel* were quantified from gels (*left panel*) using biotinylated membranes from three separate filters.

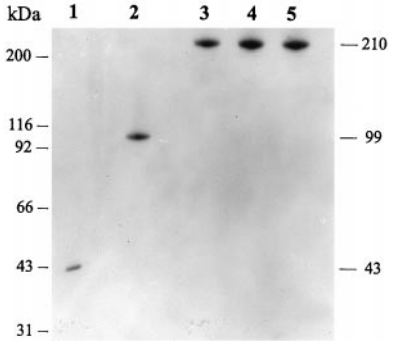

FIG. 5. **Chemical cross-linking of BLM with ^{125}I-TC II-Cbl**. ^{125}I-TC II-Cbl (30,000 dpm) was cross-linked in the presence of disuccinimidyl suberate to BLMs of filter-grown Caco-2 cells that were incubated with BFA for 12 h and then in the absence of BFA for 8 (*lane 2*), 12 (*lane 3*), and 24 h (*lane 4*). The cells were then harvested and washed, and the homogenate was subjected to nonreducing SDS-PAGE (7.5%). The bands were detected by autoradiography. The labeled ligand alone and the ligand cross-linked to BLMs of untreated cells are shown in *lanes 1* and *5*, respectively.

ligand (*lane 1*) by a receptor with a molecular mass of 56 kDa. The eventual change in the size of the cross-linked product to a molecular mass of 210 kDa is consistent with the binding of 2 mol of ligand (86 kDa/dimer) to a receptor of molecular mass of 124 kDa. Similar sized cross-linked product was also obtained by cross-linking the BLM from untreated cells (*lane 5*). These results indicated that during the reversal of BFA effects, TC

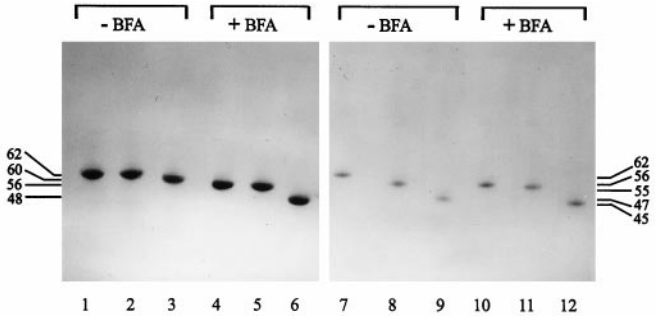

FIG. 6. **Digestion of ^{35}S-TC II-R obtained from BFA-treated and untreated cells with various glycosidases**. ^{35}S-TC II-R (20,000 dpm (*left panel*) or 5000 dpm (*right panel*)) obtained from BFA-treated (*lanes 4*, *5*, *6*, *10*, *11*, and *12*) and untreated cells (*lanes 1*, *2*, *3*, *7*, *8*, and *9*) digested with nothing (*lanes 1*, *4*, *7*, and *10*), endo-β-*N*-acetylglucosaminidase (*lanes 2* and *5*), peptide:*N*-glycosidase F (*lanes 3* and *6*), sialidase (*lanes 8* and *11*), and sialidase and *O*-glycanase (*lanes 9* and *12*) was subjected to nonreducing SDS-PAGE (7.5%). The bands were visualized by fluorography.

II-R delivered to BLM as a monomer is unable to dimerize for up to 8 h of reversal time.

The size of monomeric form of TC II-R retained in the cells following treatment with BFA was 56 kDa, while the size of BLM TC II-R from cells recovering from BFA (Fig. 4, *right panel*) increased from 56 to 62 kDa. These observations suggested that BFA treatment may inhibit terminal sialylation of the TC II-R oligosaccharides and that progressive recovery of sialylation may occur during the recovery period. In order to directly test these possibilities, the following experiments were carried out.

BFA Inhibits Sialylation of Oligosaccharides of TC II-R—The molecular mass of [^{35}S]methionine-labeled immunoprecipitated TC II-R monomer was 62 kDa in BFA-untreated cells (Fig. 6, *lanes 1* and *7*) and was 56 kDa in treated cells (*lanes 4* and *10*) cells. Following treatment with sialidase, the 62-kDa TC II-R from untreated cells was converted to 56 kDa (lane 8), while similar treatment of the 56-kDa form of TC II-R obtained from BFA-treated cells resulted in the shift equivalent to 55 kDa (*lane 11*). When the labeled receptor from both untreated (*lane 9*) and BFA-treated (*lane 12*) cells were further digested with *O*-glycanase, a shift equivalent to 9–10 kDa was noted. This observation clearly indicated that BFA-treatment did not inhibit core *O*-glycosylation of TC II-R but only the terminal sialylation of these sugars. In addition, BFA treatment also had no effect on the maturation of a single *N*-linked oligosaccharide of TC II-R, since the labeled receptors from both the untreated (*lane 1*) and treated (*lane 4*) cells were resistant to endo-β-*N*-acetylglucosaminidase (*lanes 2* and *5*) but not to peptide:*N*-glycosidase F (*lanes 3* and *6*) treatments.

Further confirmation that BFA treatment had indeed inhibited terminal sialylation of TC II-R was obtained by testing the ability of ^{35}S-TC II-R obtained from Caco-2 cells labeled in the

TABLE I
Binding of ^{35}S-TC II-R obtained from BFA-untreated and -treated cells from cells treated with BFA and then incubated with BFA-free medium to S. nigra-aggutinin-agarose

BFA-treated and -untreated cells were pulsed for 1 h with [^{35}S]methionine. Labeled TC II-R immunoprecipitate was allowed to bind to *S. nigra* agglutinin-agarose. The values reported are an average of binding using two separate labeling experiments and expressed as percentage of the radioactivity bound. The radioactivity used for binding to *S. nigra* agglutinin-agarose varied (5,000–20,000 dpm) from sample to sample, particularly when the cells were incubated in BFA-free medium. Other details are provided under "Experimental Procedures."

Treatments	Incubation time	^{35}S-TC II-R bound
	h	%
Without BFA	12	100
Without BFA, with sialidase	12	5
With BFA	12	6
With BFA-free medium	0	5
	4	7
	8	12
	12	90
	24	100

presence and absence of BFA to bind to sialic acid-specific lectin, *S. nigra* agglutinin-agarose beads. The binding of labeled TC II-R from untreated cells was 100%, and following *in vitro* digestion with sialidase, it was reduced to 5%, exactly the same level of binding noted with labeled TC II-R obtained from BFA-treated cells (Table I). However, when BFA-treated cells were incubated (0–24 h) in the absence of BFA, the binding of labeled receptor to *S. nigra* agglutinin-agarose increased only modestly from 5 to 12% between 0 and 8 h of recovery and to 90 and 100% of binding at 12 and 24 h of recovery, respectively (Table I). In order to test whether lack or incomplete sialylation of TC II-R during the early recovery periods is responsible for its failure to dimerize, pure human native placental TC II-R and enzymatically desialylated TC II-R were reconstituted using lipid bilayer prepared using total BLM lipids from untreated and BFA-treated cells and from BFA-treated cells that were allowed to recover for 8 and 12 h.

Lipid Bilayer Prepared Using BLM Total Lipids from BFA-treated Cells Does Not Support in Vitro the Dimerization of both Native and Asialo-TC II-R—Triton X-100 micellar bound sialylated (62 kDa, Fig. 7, *lane 1*) and asialo-TC II-R (56 kDa, *lane 5*) dimerized when reconstituted with bilayer prepared using total BLM lipid extract from untreated cells and from BFA-treated cells that were exposed to BFA free media for 12 h (*lanes 2* and *6*). However, both forms of TC II-R failed to dimerize when reconstituted using BLM lipid extracts from cells that were exposed to BFA-free medium for 0 (*lanes 4* and *8*) or 8 h (*lanes 3* and *7*). These results clearly indicated that lack of dimerization of TC II-R in the BLM during the early recovery periods is not due to lack or incomplete sialylation of its sugars but potentially to decreased order of BLM caused by BFA-mediated changes in the levels of PL or cholesterol or both. In order to test these possibilities, a detailed analysis of the lipid composition of the isolated BLM and the fluorescent anisotropy of the BLM using filter-grown cells was carried out.

BFA Treatment of Caco-2 Cells Decreases BLM Cholesterol Levels and Its Order without Affecting Its Total PL Levels—BFA treatment of Caco-2 cells for 12 h did not inhibit total cellular cholesterol or PL levels (Table II). After 12 h of incubation with BFA, as in untreated cells, about 8% of total cellular PL (260 ± 15 pmol) was present in the BLM. However, the percentage of total cellular cholesterol present in the BLM declined by about 90% from about 18% (95 ± 5 pmol) in untreated cells to about 2% (11 ± 1 pmol) in BFA-treated cells (Table II). When the BFA-treated cells were incubated with BFA-free medium (Table III), cholesterol levels in the BLM

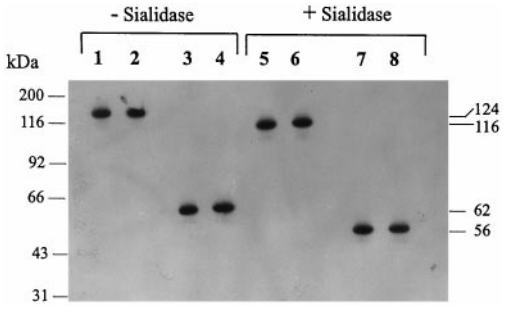

FIG. 7. **Immunoblot analysis of pure TC II-R reconstituted using total lipid extracts from the BLM of cells incubated with and without BFA and cells recovering from BFA.** Total lipid extract (2 μmol of phospholipid) from control cells (*lanes 1* and *5*) and BFA-treated cells that were recovering from BFA for 0 (*lanes 4* and *8*), 8 (*lanes 3* and *7*), and 12 h (*lanes 2* and *6*); pure sialylated native (*lanes 1–4*); and desialylated (*lanes 5–8*) pure TC II-R (0.25 μg) were reconstituted, and the liposomal pellet was subjected to immunoblot analysis as before (3).

TABLE II
Effect of BFA treatment on the total cellular and BLM cholesterol and phospholipid levels

Postconfluent Caco-2 cells grown on tissue culture flasks were incubated with BFA (5 μg/ml) for 12 h at 37 °C. The cells were then harvested for either lipid extraction to measure total PL and cholesterol or for isolating BLM. The values reported represent mean ± S.D. of triplicate assays for each lipid measurement and for lipid extracts from either cells or isolated BLM from three separate flasks. The values reported are expressed as pmol of each lipid in one flask of cells (30×10^6 cells) and in the BLM as pmol/total BLM protein/flask of cells. The values in the parenthesis represent the percentage of total cellular lipid present in the BLM. Other details are provided under "Experimental Procedures."

Treatment	Cholesterol		Phospholipid	
	Cellular	BLM	Cellular	BLM
	pmol			
Without BFA	526 ± 25	95 ± 5 (18)	3300 ± 110	260 ± 15 (8)
With BFA	495 ± 15	11 ± 1 (2)	3270 ± 95	245 ± 10 (7.4)

TABLE III
Cholesterol and phospholipid levels in the BLM of BFA-treated Caco-2 cells incubated with BFA-free medium

Caco-2 cells treated with BFA (5 μg/ml) for 12 h were washed in BFA-free medium and incubated further for the indicated times in BFA-free medium. The harvested cells were used for BLM isolation, total lipid extraction, and measuring cholesterol and phospholipid levels. The values represent mean ± S.D. from triplicate assays from BLM isolated from three separate flasks of cells and are expressed as pmol of lipid present in the BLM protein isolated from each flask. The BLM protein/flask was 208 ± 15 μg.

Incubation time	Cholesterol	Phospholipid	Phospholipid/ Cholesterol
h	*pmol*		
0	11.3 ± 1.1	248 ± 15	22.0
4	13.6 ± 0.9	230 ± 13	16.9
8	14.5 ± 1.3	240 ± 17	16.6
12	53.0 ± 3.9	210 ± 19	4.0
24	90.0 ± 5.2	235 ± 18	2.6

rose dramatically from about 14 pmol at 8 h of incubation to about 53 pmol at 12 h and 90 pmol at 24 h of incubation, respectively. The PL (Table II) and protein (data not shown) levels of the BLM did not reveal any significant changes during incubation of BFA-treated cells with BFA-free medium (Table III). Steady-state fluorescent anisotropy (which is inversely proportional to fluidity), r_s, of the BLM fell from 0.27 in BFA-untreated cells to 0.15 following 12 h of incubation of filter-grown cells with BFA for 12 h (Table IV). When the cells were exposed to BFA-free medium, the r_s value rose linearly, and in 24 h of incubation it reached normal values (Table IV).

To confirm that the BFA-induced changes noted in this

TABLE IV
Effect of BFA on the steady state fluorescent anisotropy (r_s) of the BLM

Filter-grown cells were incubated in the presence or absence of BFA (5 μg/ml) for 12 h at 37 °C. BFA-treated cells were washed with BFA-free medium and incubated in the absence of BFA for 0–24 h. The cationic probe TMA-DPH was added to the BLM. The r_s values reported are mean ± S.D. from 10 determinations at each time interval using three separate sets of filters. Other details are provided under "Experimental Procedures."

Incubation time	r_s
h	
12 (no BFA)	0.27 ± 0.02
12 (+ BFA)	0.15 ± 0.01
0 (no BFA)	0.15 ± 0.01
4	0.16 ± 0.01
8	0.19 ± 0.02
12	0.21 ± 0.01
24	0.28 ± 0.02

study, particularly the inhibition of cholesterol and TC II-R delivery to BLM, were due to Golgi disruptions, the morphological integrity of the Golgi was determined under the same experimental conditions by immunofluorescence analysis using antibody to TGN 38, an integral membrane protein that is localized predominantly to the TGN (25).

Golgi Morphology in Untreated and BFA-treated Cells and in Cells Recovering from BFA Effects—The pattern of staining in untreated cells (Fig. 8*a*) revealed intact TGN around the nucleus. However, following 12 h of treatment with BFA (Fig. 8*b*), the pattern of staining revealed complete disruption of the TGN morphology as described previously for BFA-treated cells (11, 25, 26). When the cells were allowed to recover from the effects of BFA for 24 h (Fig. 8*c*), normal TGN morphology was restored.

DISCUSSION

This study has demonstrated that during recovery (0–24 h) of Caco-2 cells from an exposure to BFA for 12 h, there was a time-dependent change in the physical state of BLM TC II-R from a monomer (0–8 h) to a dimer (12–24 h) (Figs. 3 and 5). The time frame of change (between 8 and 12 h) in the physical state of BLM TC II-R coincided with other changes in the BLM. These include increased (*a*) delivery of cholesterol (Table III), (*b*) order (Table IV), and (*c*) sialylation (Table I) and delivery of TC II-R (Fig. 4). In addition, during this period of recovery of cells from the effects of BFA, the disrupted Golgi morphology was restored (Fig. 8). Taken together, these observations lead one to conclude that BFA-mediated Golgi disruptions inhibit cholesterol delivery to BLM, thus decreasing its order and ability to support the dimerization of TC II-R. Although these conclusions are fully supported by our earlier studies (3) of *in vitro* requirements for the dimerization of TC II-R, it raises some important issues, particularly the role of the Golgi in the vesicular trafficking of cholesterol and TC II-R.

The disassociation of components of the Golgi coatomer (COP) such as β-COP and other coat proteins in most cells occur within 30 min of treatment with BFA (27–29); thus, inhibition of vesicular transport of TC II-R should occur after a shorter time of treatment with BFA. However, a 12-h treatment with BFA was needed to completely (100%) deplete BLM TC II-R activity, although 50% depletion occurred at 2 h of incubation with BFA. The prolonged time of incubation needed to completely deplete BLM of its TC II-R activity may be due to an increase in the number of rounds of TC II-R recycling from the endosomes, a decreased internalization rate, or both. It is known that PM recycling and internalization of several cell surface receptors are affected in BFA-treated cells (30, 31). In addition, since the ER to BLM transport of newly synthesized

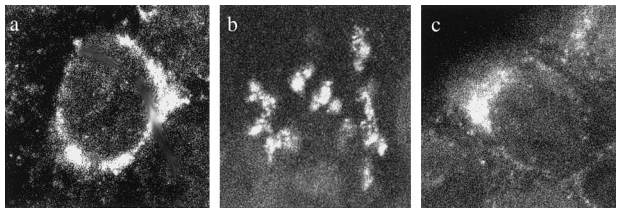

FIG. 8. **Effect of BFA treatment on morphology of the TGN.** Filter-grown Caco-2 cells were incubated in the absence (*a*) and the presence (*b*) of BFA (5 μg/ml) of BFA for 12 h at 37 °C and in the BFA-free medium for 24 h (*c*) following treatment with BFA. The cells were processed by immunofluorescence microscopy for TGN 38 staining as described under "Experimental Procedures."

TC II-R is rapid, with a $t_{1/2}$ of 15 min,[2] it is possible that more than just the disassociation of coatomers from the Golgi or collapsing of the cis- and medial stacks of the Golgi is sufficient to inhibit vesicular delivery of TC II-R to the BLM. This certainly appears to be a possibility, since a 12-h incubation with BFA had inhibited only the terminal sialylation, a step confined to the TGN (32, 33), but not the core *O*-glycosylation or maturation of its single *N*-linked oligosaccharide beyond the high mannose type, steps that occur in the early Golgi compartments. One advantage of a 12-h incubation with BFA used in this study is that the BFA effects were studied on newly synthesized TC II-R, since the $t_{1/2}$ of TC II-R in these cells is about 7.5 h (18), which remains unaltered in the presence of BFA (data not shown).

Because of the 12-h incubation of cells with BFA, its level may be drastically reduced due to its metabolic conversion, so that some of the changes noted at the end of 12 h (the inhibition of delivery of cholesterol and TC II-R to the BLM) are not due to Golgi disruptions but to other causes such as inhibition of protein and lipid synthesis or to an increased number of dying cells. However, these possibilities are highly unlikely for the following reasons. There is no evidence that incubations of Caco-2 cells with BFA for 12 h are detrimental, and the observation that all of the effects noted in our study were reversible suggests that a 12-h incubation with BFA did not permanently damage the cells. Moreover, total cellular TC II-R protein, cholesterol, and PL levels were not affected, indicating that 12-h incubation with BFA had no effect on protein or lipid synthesis. The inhibition of terminal sialylation of TC II-R in BFA-treated cells and its reversal during the recovery of cells from the effects of BFA (Table I) suggest strongly that BFA treatment disrupted the TGN sites at which sialyltranferases are localized (32, 33). Functional recovery of TGN in Caco-2 cells treated with BFA for 12 h appears to be a slow process and not needed for the delivery of TC II-R to the BLM, since asialo-TC II-R derived from the intracellularly retained pool was delivered to the BLM during the first 8 h of the recovery period. The sialylated forms of TC II-R are delivered to the BLM between 12 and 24 h of recovery time, suggesting that this form of TC II-R is derived from the newly synthesized and processed pool, when the cells have more fully recovered from the effects of BFA. The reappearance of intact TGN around the nucleus (Fig. 8*c*) following a 24-h incubation of BFA-treated cells in BFA-free medium support this suggestion.

The interesting observation that cholesterol but not PL levels of the BLM were depleted in BFA-treated Caco-2 cells raises important questions regarding the mechanism(s) by which this could occur. Since there was no change in the total cellular cholesterol levels following BFA treatment of Caco-2 cells noted in this (Table II) and a previous study (34), it is unlikely that depletion of BLM cholesterol in BFA-treated cells is due to an

[2] S. Bose and B. Seetharam, unpublished observations.

effect on the *de novo* synthesis of cholesterol. BFA treatment of Caco-2 cells for 8–24 h has been previously (34) shown to increase cholesterol ester formation. However, there is no evidence that free cholesterol that is esterified is derived from the PM pool. Thus, the most likely explanation for the depletion of BLM cholesterol is that it is due to BFA-induced morphological disruptions, the delivery of cholesterol to the BLM is inhibited.

The potential involvement of the Golgi as an intermediate in the intracellular sorting of cholesterol to the cellular PM is not fully known. Available evidence suggests that PM cholesterol is derived from both the newly synthesized pool in the ER (35) by a BFA-insensitive and thus Golgi-independent pathway (36) and from the lysosomes (37) by a BFA-sensitive, Golgi-dependent pathway (38). It is not known which one (if not both) of the two pathways operates to deliver cholesterol to BLM in Caco-2 cells and is BFA-sensitive. One could speculate that in Caco-2 cells the bulk of the BLM cholesterol is derived from the lysosomes via the Golgi, disruption of which results in increased delivery of cholesterol to the ER, where it is esterified. This speculation is supported by the following observations: (*a*) lysosomal cholesterol must pass through the PM first on its way to the ER (39) for esterification, (*b*) lysosomal cholesterol delivery to the PM is BFA-sensitive (38), (*c*) BFA treatment increases cholesterol ester formation in Caco-2 cells (34), and BFA depletes BLM cholesterol levels (Table II).

Due to decreased BLM cholesterol and unaltered PL levels in BFA-treated cells, the PL/cholesterol ratio of BLM increased from 2.6 to 22.0, thus causing a dramatic decrease in the mol % of BLM cholesterol from about 38.5% in untreated cells to 4.5% in BFA-treated cells (Table II). The depletion of BLM cholesterol levels due to disruption of its trafficking generated a more fluid or a less ordered membrane, which previously (3) has been shown not to support the *in vitro* dimerization of TC II-R. To the best of our knowledge, this finding is the first demonstration that BFA treatment of cells can deplete cholesterol levels of the PM, modulate its fluidity, and affect the physical state of a PM functional protein. Although the role of cholesterol in influencing membrane fluidity and, thus, the dimerization of TC II-R is not fully understood, previously (3) we have demonstrated that cholesterol (above the transition temperature) increases order around the 2-fatty acyl residue of PC and supports TC II-R dimerization. Taken together, our earlier *in vitro* studies (3) and the current *in vivo* studies suggest strongly that cholesterol in excess of 10 mol % through its condensing effect (40) on the fatty acyl hydrocarbon of the PL is essential for maintaining the high order of BLM and, thus, facilitates the dimerization of TC II-R.

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