

Inhibition of Secretion by 1,3-Cyclohexanebis(methylamine), a Dibasic Compound That Interferes with Coatomer Function

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We noted previously that certain aminoglycoside antibiotics inhibit the binding of coatomer to Golgi membranes *in vitro*. The inhibition is mediated in part by two primary amino groups present at the 1 and 3 positions of the 2-deoxystreptamine moiety of the antibiotics. These two amines appear to mimic the ϵ -amino groups present in the two lysine residues of the KKXX motif that is known to bind coatomer. Here we report the effects of 1,3-cyclohexanebis(methylamine) (CBM) on secretion *in vivo*, a compound chosen for study because it contains primary amino groups that resemble those in 2-deoxystreptamine and it should penetrate lipid bilayers more readily than antibiotics. CBM inhibited coatomer binding to Golgi membranes *in vitro* and *in vivo* and inhibited secretion by intact cells. Despite depressed binding of coatomer *in vivo*, the Golgi complex retained its characteristic perinuclear location in the presence of CBM and did not fuse with the endoplasmic reticulum (ER). Transport from the ER to the Golgi was also not blocked by CBM. These data suggest that a full complement of coat protein I (COPI) on membranes is not critical for maintenance of Golgi integrity or for traffic from the ER to the Golgi but is necessary for transport through the Golgi to the plasma membrane.

INTRODUCTION

Coat protein I (COPI) is a protein complex concentrated on membranes of the intermediate compartment (also called vesicular tubular clusters, the endoplasmic reticulum-Golgi-intermediate compartment, the salvage compartment, and the 15°C compartment) and the Golgi complex in mammalian cells (Oprins *et al.*, 1993; Pind *et al.*, 1994; Griffiths *et al.*, 1995). Surprisingly, COPI was also found recently on peroxisomes (Passreiter *et al.*, 1998). COPI consists of ADP-ribosylation factor 1 (ARF1)¹ and coatomer (for reviews, see

Kreis and Pepperkok, 1994; Kreis *et al.*, 1995; Rothman and Wieland, 1996). ARF1, a small GTP-binding protein, is implicated in recruiting coatomer to membranes. Coatomer is a soluble macromolecular protein complex that contains seven protein subunits (α , β , β' , γ , δ , ϵ , and ζ). The precise role of COPI-coated vesicles in secretory membrane traffic is under debate (for reviews, see Bannykh and Balch, 1997; Cosson and Letourneur, 1997; Schekman and Mellman, 1997). Strong genetic and biochemical evidence indicates that COPI-coated vesicles function in retrograde transport, conveying membrane from the Golgi complex back to the endoplasmic reticulum (Letourneur *et al.*, 1994; Cosson *et al.*, 1996; Lewis and Pelham, 1996). Biochemical and morphological evidence also suggests that COPI-coated vesicles function in anterograde transport, conveying membrane through the Golgi complex toward the plasma membrane (Fiedler *et al.*, 1996; Orci *et al.*, 1997). Thus, COPI may have a dual function and partic-

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¹ Abbreviations used: ARF1, ADP-ribosylation factor 1; BFA, brefeldin A; CBM, 1,3-cyclohexanebis(methylamine); CHO, Chinese hamster ovary; COPI, coat protein I; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin of influenza virus; ManII, mannosidase II; NRK, normal rat kidney; TGN, trans-Golgi network; VSV, vesicular stomatitis virus.

ipate in both anterograde and retrograde membrane transport.

Coatomeer binds peptides containing the carboxy-terminal motif KKXX in which K is lysine and X is any amino acid (Cosson and Letourneur, 1994). This motif, or a closely related sequence, is present in the cytoplasmic domains of many type I transmembrane proteins that are residents of the endoplasmic reticulum and is a signal for returning the proteins to the endoplasmic reticulum should they escape to the Golgi complex (Jackson *et al.*, 1990, 1993). The fact that coatomeer binds this retrieval motif is part of the evidence that COPI-coated vesicles function in retrograde transport from the Golgi to the endoplasmic reticulum. Coatomeer was also recently reported to bind peptides containing a diphenylalanine motif, and it was suggested that this interaction may mediate anterograde transport by COPI (Fiedler *et al.*, 1996; Fiedler and Rothman, 1997). The basis for anterograde or retrograde selectivity of COPI might be related to sequence motifs in transmembrane cargo proteins.

Coatomeer recruitment to membranes involves the GTPase cycle of ARF1, including the action of a guanine nucleotide exchange factor that promotes the exchange of GDP for GTP and results in the association of cytoplasmic ARF1 with membranes. How the binding of ARF1 to membranes recruits coatomeer is not well understood, but ARF1 can be cross-linked to the β subunit of coatomeer (Zhao *et al.*, 1997), suggesting that coatomeer could bind ARF1 directly on the membrane. Alternatively, ARF1 may promote coatomeer binding to another receptor on the membrane. In addition, ARF1 stimulates phospholipase D (Ktistakis *et al.*, 1995), and there is evidence that ARF1 need not be present for coatomeer binding provided phospholipase D is activated (Ktistakis *et al.*, 1996).

Studies with the drug brefeldin A have strongly influenced models of coatomeer function. Brefeldin A dissociates COPI from membranes of intact cells and induces the Golgi apparatus and the endoplasmic reticulum (ER) to fuse, suggesting that removal of the COPI coat might induce membrane fusion. However, the molecular mechanism of brefeldin A action is not well understood, and recent work of Mironov *et al.* (1997) suggests that the dissociation of coatomeer from Golgi membranes induced by brefeldin A may not be sufficient to cause unregulated membrane fusion. It would be useful to have alternative agents that interfere with coatomeer binding in intact cells to help investigate coatomeer function and to compare the consequences with those of brefeldin A treatment. We recently found that certain aminoglycoside antibiotics bind to coatomeer *in vitro* (Hudson and Draper, 1997). The features of this interaction suggested that the antibiotics were binding to the same site on coatomeer that binds the KKXX sequence and that there were at least two such sites per coatomeer. Guided by the struc-

ture of the antibiotics, we studied compounds that might retain the ability to interact with coatomeer and also more readily pass through the plasma membrane so that the effects of the compounds on the secretory process could be analyzed with intact cells. We found that 1,3-cyclohexanebis(methylamine) (CBM) inhibited coatomeer binding to membranes *in vitro* and *in vivo* and inhibited secretion by intact cells. However, the drug did not induce the Golgi apparatus to fuse with the ER and did not inhibit transport from the ER to the Golgi.

MATERIALS AND METHODS

Reagents

L-1-Tosylamide-2-phenylethylchloromethyl-trypsin, soybean trypsin inhibitor, protein A-Sepharose CL-4B, endoglycosidase H, HEPES, sucrose, magnesium acetate, potassium chloride, ATP, GTP, creatine phosphate, creatine phosphokinase, protease inhibitors, di-lysine, and superfibronectin were purchased from Sigma Chemical (St. Louis, MO). Brefeldin A was purchased from Calbiochem (La Jolla, CA), dissolved in DMSO to 1 mg/ml, and stored at -20°C before use. Tran ^{35}S label was from ICN Radiochemical (Irvine, CA). CBM, 1,2-diaminocyclohexane, and 1,3-diaminopropane were from Acros Organics (Pittsburgh, PA). Fluoromount G was from Fisher Scientific (Pittsburgh, PA).

Antibodies

Mouse anti-vesicular stomatitis virus (VSV) G protein (monoclonal P5D4) and horseradish peroxidase conjugated either to goat anti-mouse IgG or to protein A were from Sigma Chemical. Goat anti-hemagglutinin of influenza virus (-HA) (strain X31) antiserum was from Dr. R. Webster (Saint Jude Children's Hospital, Memphis, TN). Rabbit anti-mannosidase II was from Dr. K. Moremen (The University of Georgia, Athens, GA). Anti- β -COP monoclonal antibody M3A5 was obtained from the supernatant of hybridoma cells. The cells were provided by Dr. Thomas Kreis (Universite de Geneve, Geneva, Switzerland). Mouse anti-trans-Golgi network (-TGN)38 monoclonal antibody (mAb2F 7.1) was from Affinity Bioreagents (Golden, CO). Mouse anti- γ -adaptin monoclonal antibody (mAb 88) was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-VSV G protein was prepared with G protein as the immunogen.

Cells and Virus

The propagation and use of recombinant influenza virus X31 were as described by Wang *et al.* (1990). Cells were cultured as described previously (Kao and Draper, 1992; Bau and Draper, 1993).

Analysis of Influenza Virus HA Protein Transport

To study the effect of diamino compounds on the transport of influenza virus HA protein from the ER to the Golgi and then to the cell surface, we grew Chinese hamster ovary (CHO) or normal rat kidney (NRK) cells to confluence in 24-well plates 1 d before an experiment. Cells were infected with influenza virus at 37°C for 45 min, washed, and incubated for a further 3.5–4 h. The cells were then incubated for 30 min in assay medium (DMEM lacking methionine and sodium bicarbonate and supplemented with 10% dialyzed fetal bovine serum and diamino compounds, pH 8.8). Subsequently, cells were incubated for 5 min with assay medium containing 100 $\mu\text{Ci/ml}$ Tran ^{35}S label. The radioactive medium was then removed, and 0.2 ml of fresh assay medium was added for further incubation.

To assess the acquisition of endoglycosidase H resistance by HA and the sensitivity of HA to extracellular trypsin, the labeled cells were rinsed once with PBS and treated on ice with PBS containing 100 $\mu\text{g}/\text{ml}$ L-1-tosylamide-2-phenylethylchloromethyl-trypsin for 30 min. Ten microliters of 10 mg/ml soybean trypsin inhibitor were added 15 min before the cells were lysed with lysis buffer (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 0.1 $\mu\text{g}/\text{ml}$ aprotinin, and 0.1 mg/ml soybean trypsin inhibitor). HA was immunoprecipitated with goat anti-HA antibody and protein A-Sepharose 4B. The immunoprecipitated complex was boiled for 5 min in 50 mM sodium citrate, pH 5.5, containing 0.1% SDS. Two 10- μl aliquots were taken from each sample. One aliquot was mixed with 10 μl of 50 mM sodium citrate, pH 5.5, containing 0.5 mU of endoglycosidase H, whereas the other one was mixed with citrate buffer only. After incubation at 37°C for 16–20 h, each sample was mixed with Laemmli sample buffer and electrophoresed in a 10.5% SDS-polyacrylamide gel. Radiolabeled protein bands were scanned and quantitated with the STORM PhosphorImager system from Molecular Dynamics (Sunnyvale, CA). The following formula was used to determine the fraction of HA proteins processed to the endoglycosidase H-resistant form (Endo H_R) or the trypsin-sensitive form (Trypsin_S):

$$\text{Endo H}_R (\%) = 100 \times [(\text{HA0 Endo H}_R + \text{HA1} + \text{HA2})/\text{HA total}];$$

$$\text{Trypsin}_S (\%) = 100 \times [(\text{HA1} + \text{HA2})/\text{HA total}];$$

$$\text{HA total} = \text{HA0 Endo H}_S + \text{HA0 Endo H}_R + \text{HA1} + \text{HA2}$$

Binding of Coatomer to Golgi-enriched Membranes

Golgi-enriched membranes and cytosol were prepared as detailed previously (Hudson and Draper, 1997). The binding of coatomer in cytosol to Golgi-enriched membranes was also measured as described by Hudson and Draper (1997). Briefly, membranes (5 μg) and cytosol (60 μg) were incubated with 1 mM ATP, 1 mM GTP, 5 mM creatine phosphate, 8 U of creatine phosphokinase, and protease inhibitors in a total volume of 100 μl for 20 min at 34°C. Binding reactions were stopped by chilling to 4°C, and membranes were collected by centrifugation at 16,000 $\times g$ for 10 min at 4°C in a microfuge. The pelleted membranes were dissolved in sample buffer and electrophoresed in 7% polyacrylamide gels with SDS. Proteins were transferred to nitrocellulose by electrophoresis, and the nitrocellulose was incubated with 0.1% Tween 20 as a blocking agent, followed by incubation with monoclonal antibody M3A5 to β -COP. The secondary antibody for detecting β -COP was horseradish peroxidase-conjugated goat anti-mouse. The blots were developed with the Pierce Chemical (Rockford, IL) Supersignal ECL system. Films were digitized using an LKB (Piscataway, NY) densitometer, and the intensities of bands were quantitated using ImageQuant (Molecular Dynamics, Sunnyvale, CA) analysis software.

Analysis of G Protein Glycosylation

CHO cells were infected with the ts045 strain of VSV at 40°C and then radiolabeled as described for influenza virus. To determine the acquisition of endoglycosidase H resistance of the G protein, we rinsed the labeled cells once with PBS and then lysed the cells with lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 0.1 $\mu\text{g}/\text{ml}$ aprotinin, and 0.1 mg/ml soybean trypsin inhibitor). G protein was immunoprecipitated with rabbit anti-G protein antibody and protein A-Sepharose 4B. The immunoprecipitated complex was then treated with endoglycosidase H and electrophoresed with SDS in an 8.0% polyacrylamide gel.

Immunofluorescence Microscopy

NRK cells were plated on glass coverslips in the presence of 1 $\mu\text{g}/\text{ml}$ superfibronectin 1 d before the experiments. The presence of

superfibronectin was important because CBM had a tendency to reduce adherence of cells to surfaces. In all of the indirect immunofluorescence experiments, cells were fixed and permeabilized in cold methanol for 15 min, rinsed three times with PBS, and then blocked for 10 min with 1% BSA in PBS. Cells were incubated with primary antibody for 30 min at room temperature followed by rinsing and blocking procedures as just described. The same incubation and washing procedure was applied to secondary antibody. Rabbit antisera were detected with an FITC goat anti-rabbit IgG, and mouse antisera were detected with TRITC goat anti-mouse IgG. Coverslips were mounted in Fluoromount G and viewed with a Zeiss (Thornwood, NY) photoscope equipped with epifluorescence illumination and a 40 \times Apochromat lens. Photography was with Kodak (Rochester, NY) TMAX 400 film.

Protein Synthesis

The effect of diphtheria toxin on protein synthesis was measured by assessing the incorporation of radioactivity from Tran³⁵S label into acid insoluble protein as described by Bau and Draper (1993) with modifications. Cells were plated at 10⁵ cells per well in 48-well culture dishes the day before an experiment. The cells were incubated in DMEM, pH 8.8, lacking methionine and bicarbonate in the presence of the desired concentration of CBM for 15 min at 37°C. Diphtheria toxin was added, and the cells were incubated for another 90 min. Tran³⁵S label was added for 15 min, and the cells were washed, lysed, and spotted on filter paper. The filter paper was incubated in 5% trichloroacetic acid containing 0.5 mg/ml methionine for 30 min at room temperature and dried, and the radioactivity associated with each cell lysate was measured with the PhosphorImager.

RESULTS

The Effect of Antibiotics and Structural Analogues on Secretion

We noted previously that certain aminoglycoside antibiotics precipitated coatomer from cytosol and also prevented the binding of coatomer to Golgi membranes *in vitro* (Hudson and Draper, 1997). The functional groups on the antibiotics that interacted with coatomer were pairs of amino groups that appeared to mimic the ϵ -amino groups of the lysine residues in the KKXX retention motif. It follows that the antibiotics might interfere with secretion by intact cells if they block coatomer binding to membranes in the cytosol, and we tested this idea by studying the effect of Geneticin on secretion. We observed a 50% inhibition of secretion in the presence of 25 mM Geneticin; however, this high concentration of Geneticin also strongly inhibited protein synthesis (our unpublished observations). We therefore turned our attention to identifying drugs that should maintain the structural features necessary to interact with coatomer but that would enter cells more readily and also not strongly inhibit protein synthesis.

A core component of aminoglycoside antibiotics, including Geneticin, is 2-deoxystreptamine, whose structure is shown in Figure 1. 2-Deoxystreptamine contains a pair of amino groups and also appears to interact with coatomer via these groups *in vitro* (Hudson and Draper, 1997). We studied three compounds

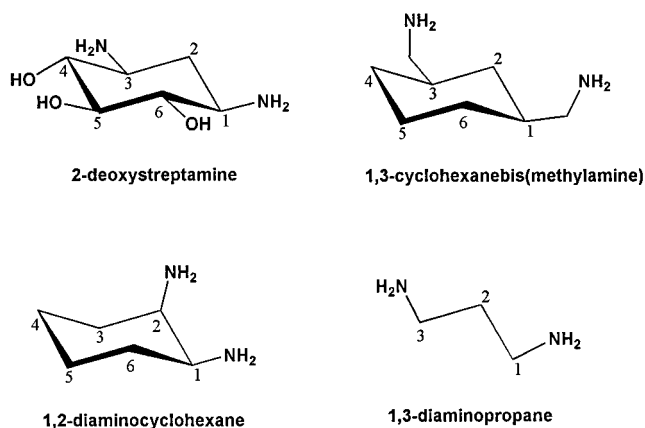


Figure 1. Structures of diamino compounds used in this study.

structurally similar to 2-deoxystreptamine: CBM, 1,2-diaminocyclohexane, and 1,3-diaminopropane (Figure 1). CBM and 1,2-diaminocyclohexane contain two primary amino groups and also retain a cyclohexyl ring structure; however, they lack hydroxyl groups, which should facilitate passage through membranes. 1,3-Diaminopropane is the simplest analogue of 2-deoxystreptamine, but the amino groups are not constrained by the cyclohexyl structure and should have more conformational flexibility.

The HA protein of influenza virus was used as the model system to monitor secretion quantitatively. Cells were infected with influenza virus and exposed to different concentrations of the three drugs. The pH of the medium during exposure to drugs (and in control cells not exposed to drugs) in this and subsequent experiments was 8.8 to facilitate the entry of the basic drugs into cells. After a pulse and chase with radioactive methionine, the amount of the HA protein on the cell surface was assessed by exposing intact cells to trypsin. Only HA transported to the cell surface is cleaved by trypsin in intact cells to produce the products HA1 and HA2, which can be separated from uncleaved, intact HA by electrophoresis in polyacrylamide gels with SDS. Thus, the percent of total HA sensitive to trypsin digestion is a measure of secretion. Both CHO and NRK cells were included in the study, and we also measured the effects of the drugs on protein synthesis with CHO cells. CBM strongly inhibited HA secretion in both CHO and NRK cells in the range of 1–1.5 mM (Figure 2, top). The drug also had a dose-dependent effect on protein synthesis, but it is evident that the inhibition of secretion occurred at low concentrations of drug that had little effect on protein synthesis. The effect of CBM on protein synthesis varied with different lots and was sometimes as high as 80% inhibition at 2 mM and above; nevertheless, all lots of CBM strongly inhibited secretion at concentrations at which there was a minimal effect on

protein synthesis. 1,2-Diaminocyclohexane also inhibited secretion, but not as effectively as did CBM (Figure 2, middle). 1,3-Diaminopropane had little effect on either protein synthesis or secretion even at the highest concentration tested of 10 mM (Figure 2, bottom). The remainder of this study focused on CBM, the most effective drug.

CBM Inhibits Coatomer Binding to Golgi Membranes In Vitro and In Vivo

CBM contains two primary amino groups that are predicted to specify interaction with the dilysine-binding sites on coatomer. If so, CBM should inhibit the binding of coatomer to Golgi membranes in vitro, analogous to the effects of dilysine (Hudson and Draper, 1997). To test this, Golgi membranes and cytosol were incubated together under conditions to promote coating, and the effects of dilysine (a positive control) and CBM on the extent of coating were measured. Coating was assessed by immunoblotting for the β -COP subunit of coatomer associated with pelleted membranes. The immunoblot is shown in Figure 3 with a quantitation of the β -COP bands given below the blot. In the absence of either Golgi membranes or cytosol, there was very little β -COP detected, demonstrating the necessity of these components for the coating reaction (Figure 3, lanes 1 and 2). The level of coating with the complete reaction mixture is shown in Figure 3, lane 3, and this reaction is taken as 100% in the quantitation below the blot. Enhanced coating was observed in the presence of GTP γ S, a nonhydrolyzable analogue of GTP (Figure 3, lane 4), as expected. Dilysine stimulated coating at 0.5 mM but then inhibited coating at higher concentrations (Figure 3, lanes 5–7). The stimulation of coating by low concentrations of a diamino compound, followed by inhibition at higher concentrations, is an effect we have frequently observed and is also evident in our previously published data (Hudson and Draper, 1997). CBM also stimulated coating at low concentrations but then strongly inhibited coating at 10 mM and above (Figure 3, lanes 8–11). We conclude that CBM is similar to dilysine and aminoglycoside antibiotics that interact with coatomer via dilysine-binding sites and in so doing CBM interferes with coatomer binding to Golgi membranes.

We also checked whether 1,3-diaminopropane inhibited coatomer binding to Golgi membranes in vitro and found no inhibition up to 20 mM, the highest concentration tested (our unpublished observations). This is consistent with the observation that 1,3-diaminopropane did not inhibit HA secretion and suggests that there is something specific to the structure of CBM beyond its dibasic nature that is critical for the interference of coatomer binding to Golgi membranes.

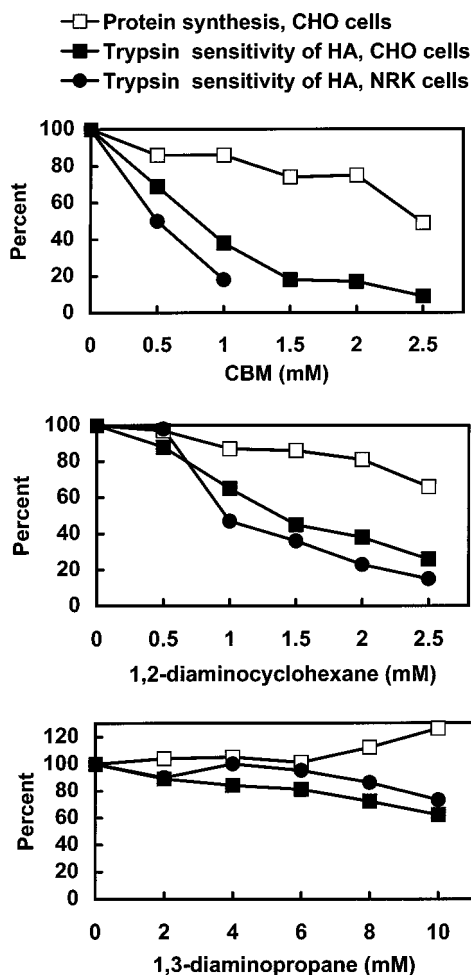


Figure 2. Effects of diamino compounds on protein synthesis and HA transport in CHO and NRK cells. To measure the extent of secretion by the trypsin sensitivity of HA, cells were infected with influenza virus and exposed to the indicated concentrations of drugs for 30 min at 37°C. The cells were incubated with Tran^{35}S label for 5 min and then incubated for 1 h in the absence of radioactive medium. The sensitivity of HA to trypsin was assessed as described in MATERIALS AND METHODS. To measure protein synthesis, uninfected cells were exposed to drugs and incubated for 1.5 h at 37°C. Tran^{35}S label was added during the last 30 min. The cells were lysed, and acid-insoluble radioactivity was measured.

To determine whether CBM reduced coatomer binding to the Golgi complex of intact cells, NRK cells were treated with CBM for 90 min and prepared for double immunofluorescence using antibodies to β -COP and to a marker for the medial Golgi, mannosidase II. Figure 4, right, demonstrates that the fluorescent signal associated with COPI on Golgi membranes was diminished at 1 mM CBM and strongly reduced at 2 mM CBM. Thus, CBM appears to reduce coatomer binding to the Golgi both *in vitro* and *in intact cells*. 1,3-Diaminopropane did not reduce coatomer binding to the Golgi complex of NRK cells (our unpublished

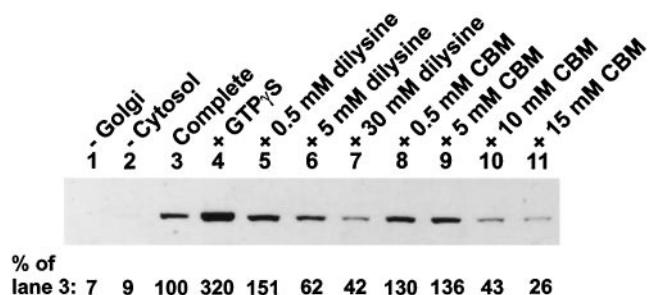


Figure 3. Effect of CBM on the binding of β -COP to Golgi-enriched membranes. Golgi-enriched membranes and cytosol were incubated together (Complete) under conditions to promote coatomer binding as described in MATERIALS AND METHODS. Omissions (-) and additions (+) to the reaction mixtures are indicated. Membrane-associated β -COP was detected by immunoblotting. The numbers below the bands represent a quantitative analysis of the band intensities as a percent of the complete reaction mixture (lane 3).

observations). It is interesting, however, that the reduced COPI signal was not accompanied by a disappearance of the Golgi complex, as occurs in response to brefeldin A. There was a swelling and disorganization of the fine reticular structure of the Golgi complex, but mannosidase II retained a definite perinuclear location (Figure 4, left). This result suggests that the Golgi complex can maintain a morphologically identifiable structure despite a strong reduction in the amount of bound COPI.

CBM is a weak base and has the potential to elevate the mildly acidic pH within compartments of the Golgi complex. Such an increase in pH could conceivably cause COPI to dissociate from Golgi membranes. To check this, NRK cells were incubated with 10 mM ammonium chloride, which is known to raise the pH within acidic organelles (de Duve *et al.*, 1974), and the distribution of β -COP was assessed by immunofluorescence microscopy. Despite an obvious swelling of lysosomes caused by ammonium ion accumulation, there was no reduction in the β -COP signal coincident with the Golgi complex (our unpublished observations). This suggests that the effect of CBM on COPI dissociation from the Golgi complex is not correlated with changes in luminal Golgi pH. Additionally, incubating the cells with 10 mM 1,3-diaminopropane also had no effect on the distribution of β -COP (our unpublished observations).

It is difficult to quantitate from immunofluorescence micrographs such as Figure 4 the extent to which CBM removes COPI from Golgi membranes *in vivo*. The difficulty is exacerbated by the fact that the Golgi complex remains in place and is a source of background fluorescence. To approach the question of how effectively CBM removed COPI from Golgi membranes, we examined the influence of AlF_4^- on COPI distribution in the presence of CBM. AlF_4^- stabilizes coatomer binding to Golgi membranes *in vitro* and *in*

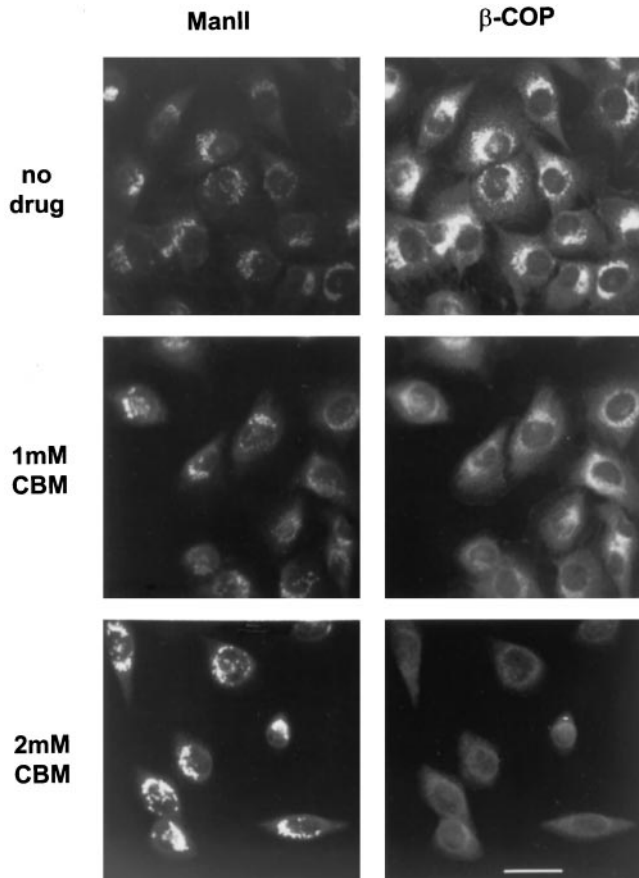


Figure 4. Distribution of mannosidase II and β -COP in CBM-treated cells. NRK cells grown on glass coverslips were incubated with the indicated concentration of CBM at 37°C for 90 min. The cells were then fixed and double-labeled with antibodies to mannosidase II (ManII; left) and β -COP (right) followed by FITC- and TRITC-coupled secondary antibodies as described in MATERIAL AND METHODS. Bar, 10 μ m.

vivo and renders COPI highly resistant to removal by brefeldin A, apparently by inhibiting COPI dissociation (Donaldson *et al.*, 1991a; Finazzi *et al.*, 1994). The mechanism by which AlF_4^- stabilizes COPI binding is not well understood but probably involves trimeric G proteins (Donaldson *et al.*, 1991a; Helms *et al.*, 1998). If coatomer retains significant ability to bind Golgi membranes in the presence of CBM, then COPI should accumulate on the Golgi in the presence of AlF_4^- , because of inhibition of COPI dissociation. In a control experiment to assess the effectiveness of AlF_4^- , cells were treated with AlF_4^- for 15 min, followed by brefeldin A for 15 min (Figure 5, top). As noted by Donaldson *et al.* (1991a), AlF_4^- stabilized COPI association with the Golgi, and brefeldin A was unable to promote β -COP dissociation or the disappearance of the Golgi. When cells were first treated with brefeldin A for 15 min, followed by AlF_4^- for 15 min (Figure 5, middle), there was no significant staining for either mannosidase II or β -COP, verifying that the Golgi complex was gone (fused with the ER) and that AlF_4^- did not reverse this action. After cells were treated for 45 min with CBM, followed by the addition of AlF_4^- for 45 min, the Golgi was still evident, but there was still strongly reduced staining of β -COP associated with the Golgi (Figure 5, bottom). The fact that AlF_4^- could not induce β -COP to accumulate on Golgi membranes in the presence of CBM argues that CBM is highly effective in blocking coatomer binding to membranes.

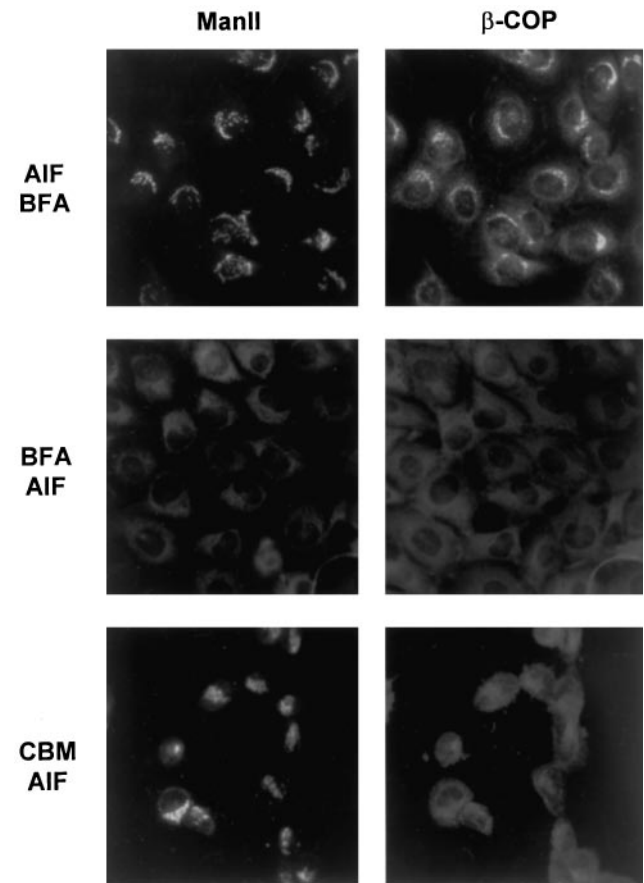


Figure 5. The influence of AlF_4^- on the distribution of mannosidase II and β -COP in the presence of CBM. AlF_4^- was prepared by adding 30 mM NaF to medium (pH 8.8) followed by the addition of 100 μ M AlCl_3 . Top, cells were treated with medium containing AlF_4^- for 15 min, and brefeldin A (BFA, 1 μ g/ml) was added for 15 min in the presence of AlF_4^- . Middle, cells were treated with brefeldin A (1 μ g/ml) for 15 min, and AlF_4^- was added for 15 min in the presence of brefeldin A. Bottom, cells were incubated with CBM (2 mM) for 45 min, and AlF_4^- was added for 45 min in the presence of CBM. Samples were prepared for immunofluorescence microscopy with antibodies to mannosidase II (left) and β -COP (right) as described in Figure 4.

CBM Does Not Induce Golgi Enzymes to Enter the ER

CBM and brefeldin A have in common that both impair coatomer binding to Golgi membranes, although by different mechanisms, and also block secretion. In addition, brefeldin A induces the fusion of Golgi

membranes with the ER, which results in the absence of an identifiable Golgi complex in cells. However, the results in Figures 4 and 5 suggest that CBM does not induce the Golgi complex to enter the ER despite the reduction in bound COPI. To verify the latter finding, we assessed whether or not Golgi enzymes entered the ER in the presence of CBM by whether the N-linked carbohydrate chains of a glycoprotein in the ER showed evidence of modification by Golgi enzymes. CHO cells were infected with the ts045 strain of VSV at the restrictive temperature to retain the G protein within the ER. The cells were then treated either with no drugs, with brefeldin A (a positive control), or with 1.5 mM CBM. Thirty minutes after drug addition, the cells were pulsed with radioactive methionine, and the sensitivity of the G protein in the ER to digestion by endoglycosidase H was determined at times after the pulse (Figure 6A). In the absence of drugs, the G protein remained sensitive to digestion by endoglycosidase H, as expected for a protein within the ER (Figure 6B, top). In the presence of brefeldin A, the G protein was fully resistant to digestion, indicating that enzymes required for carbohydrate processing of glycoproteins in the Golgi complex had been relocated to the ER under the influence of brefeldin A (Figure 6B, middle) (Doms *et al.*, 1989). In contrast, the G protein remained sensitive to endoglycosidase H in cells treated with 1.5 mM CBM (Figure 6B, bottom). Similar results were obtained with CBM at 1.0 and 2.0 mM (our unpublished observations). These data support the conclusion that CBM does not cause the Golgi complex to fuse with the ER, implying that CBM and brefeldin A operate in fundamentally different ways, despite evidence that both drugs prevent coatamer binding to the Golgi.

CBM Blocks Transport from the Golgi to the Plasma Membrane but Not from the ER to the Golgi

Considering that CBM inhibits secretion in a way that appears to be different from that of brefeldin A, we determined whether the block in secretion by CBM was at the level of the ER or the Golgi. Cells were infected with influenza virus and exposed either to no drug or to CBM. After a pulse with radioactive methionine, intact cells were treated with trypsin to cleave secreted HA on the cell surface. Total HA was then extracted from cells and treated with endoglycosidase H to determine the percent of HA that was resistant to digestion and therefore in the Golgi complex. Figure 7A shows the various radioactive HA bands in the gel under the conditions of the experiment. A quantitative analysis of these bands with the PhosphorImager was used to calculate the data presented in Figure 7, B and C. The rate of HA secretion in the presence and absence of CBM is given by the rate at which HA acquires sensitivity to trypsin (Figure 7B), and the data

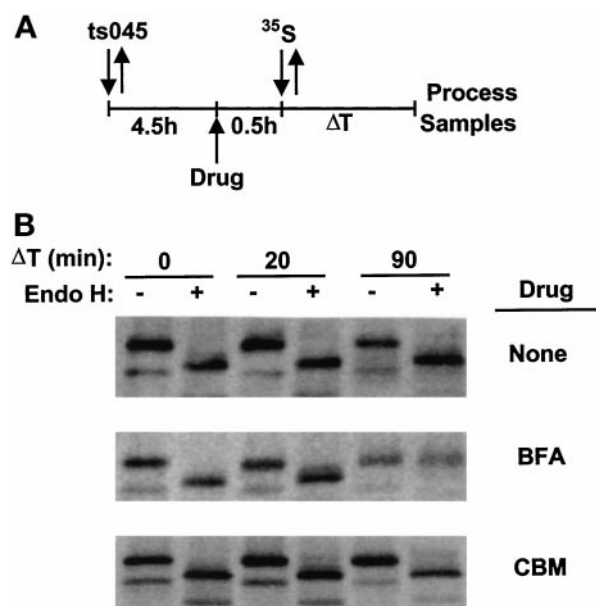


Figure 6. CBM does not cause redistribution of Golgi proteins to the ER. (A) A summary of the procedure. Cells were infected with the ts045 strain of VSV at 40°C and then were exposed for 30 min either to no drugs, to brefeldin A (0.5 μg/ml), or to CBM (1.5 mM). Tran³⁵S label was added for 15 min, and the incubation with or without drugs was continued for the indicated times. The cells were then lysed, and the G protein was immunoprecipitated, treated with endoglycosidase H, and electrophoresed as described in MATERIALS AND METHODS. (B) The migration of the G protein under the various conditions of the experiment.

verify that CBM inhibits transport to the cell surface. The rate of HA export from the ER to the Golgi is given by the rate at which HA acquires resistance to endoglycosidase H (Figure 7C). This is a valid measurement of HA export because the data in Figure 6 demonstrate that the acquisition of endoglycosidase H resistance is not caused by the return of Golgi enzymes to the ER. It is evident that CBM slightly delays export from the ER but does not impair the complete export of HA from the ER to the Golgi.

In another approach to address where CBM blocks secretion, the effect of the drug on transport of the G protein of VSV strain ts045 was assessed by immunofluorescence microscopy. Cells were infected with virus for 4 h at 41°C to trap the temperature-sensitive G protein in the ER. Medium at pH 8.8 was added with or without 2 mM CBM, and the cells were incubated for an additional 10 min at 41°C, followed by a 2 h incubation at 32°C to activate secretion of the G protein. Immunofluorescence microscopy revealed that the G protein was primarily on the cell surface in the absence of CBM but was retained in a perinuclear location coincident with the Golgi complex in the presence of CBM (our unpublished observations). Thus, the G protein left the ER in the presence of CBM but

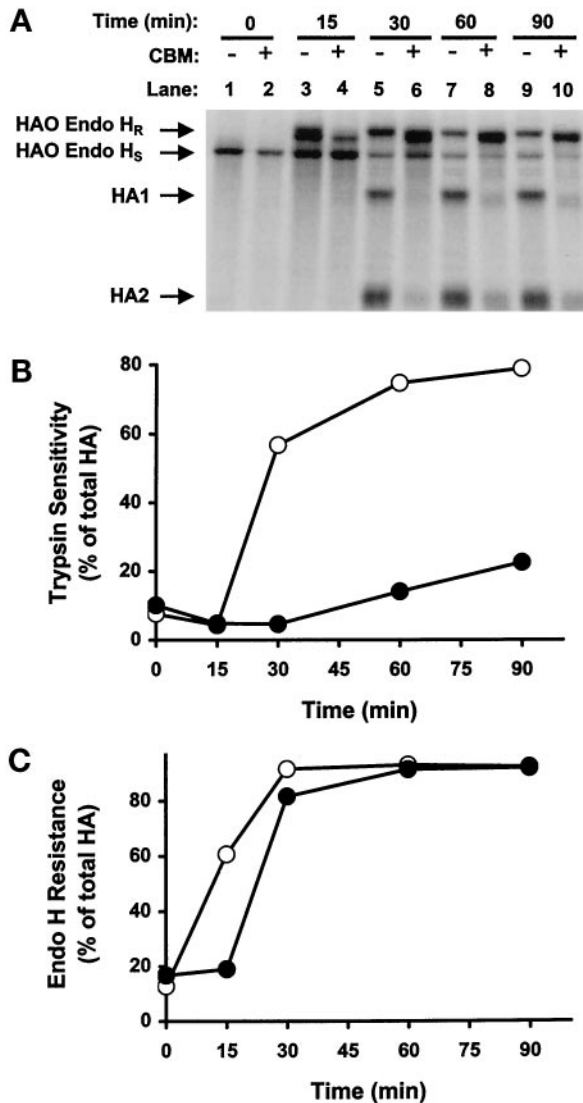


Figure 7. The effects of CBM on the transport of HA from the ER to the Golgi and from the Golgi to the plasma membrane in CHO cells. Influenza virus-infected CHO cells were either untreated (B and C, ○) or treated with CBM (B and C, ●), radiolabeled, and then chased for the indicated times. After trypsin treatment, the cells were solubilized, and HA molecules were immunoprecipitated, treated with endoglycosidase H, and resolved in 10.5% SDS-polyacrylamide gels (A). Transport to the plasma membrane (B) and to the Golgi (C) was determined by trypsin sensitivity and endoglycosidase H resistance, respectively, as described in MATERIALS AND METHODS.

was unable to exit the Golgi region, consistent with data on HA.

Data presented so far indicate that CBM does not block transport from the ER to the Golgi and that the morphology of the Golgi complex is also maintained in the presence of the drug. To verify these results in a different experiment, we incubated NRK cells with

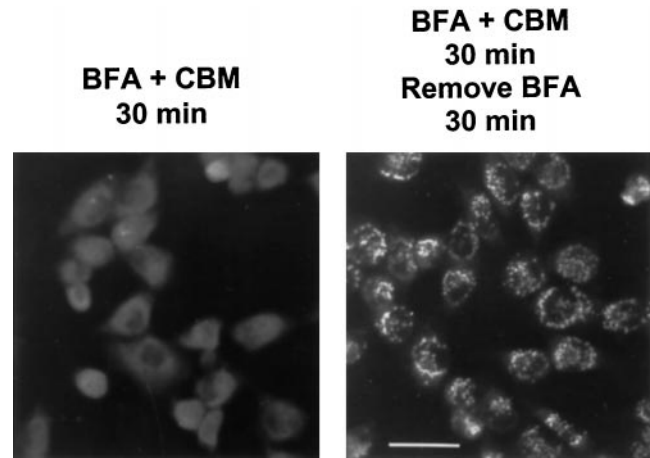


Figure 8. Reformation of the Golgi complex in cells first treated with brefeldin A and CBM followed by removal of the brefeldin A but retention of the CBM. NRK cells grown on glass coverslips were incubated with 2 mM CBM and 5 μg/ml brefeldin A for 30 min. Brefeldin A was then removed, but 2 mM CBM was retained. After a further 30 min incubation, the cells were fixed and prepared for immunofluorescence microscopy with anti-mannosidase II antibody.

both brefeldin A and 2 mM CBM. After 30 min in the presence of both drugs, the Golgi was no longer evident (Figure 8, left), indicating that CBM did not inhibit the ability of brefeldin A to induce fusion of the Golgi and the ER. The brefeldin A was removed, but the CBM maintained, to see whether the Golgi complex could reform in the presence of CBM. Thirty minutes after removal of the brefeldin A, mannosidase II had left the ER and was present in perinuclear structures that resembled the Golgi complex despite the presence of CBM (Figure 8, right). These morphological data support the conclusion that CBM does not block export from the ER; otherwise the Golgi would not have reformed.

CBM Inhibits the Association of γ -Adaptin with the TGN

We also studied the effect of CBM on the distribution of γ -adaptin on the TGN in cells by immunofluorescence microscopy. CBM significantly reduced the intensity of γ -adaptin staining associated with the Golgi complex (Figure 9, right) and again had little effect on the morphology of the Golgi as indicated by mannosidase II staining. To see whether the reduction in γ -adaptin stain was accompanied by a change in the morphology of the TGN itself, we measured the effect of CBM on the staining of TGN38, a marker for the TGN. There appeared to be a slight disorganization of the TGN at 2 mM CBM, but an identifiable TGN was still evident (Figure 10, right). Thus, it seems that CBM treatment reduces the amount of γ -adaptin on the

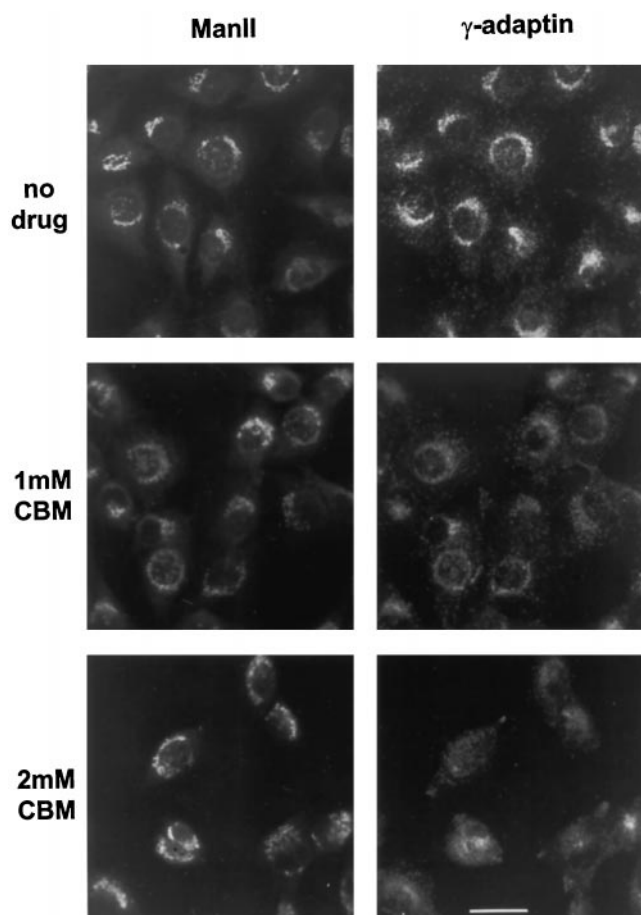


Figure 9. Distribution of mannosidase II and γ -adaptin in CBM-treated cells. NRK cells were prepared for immunofluorescence microscopy as described in Figure 4 except that the cells were double-labeled with antibodies to mannosidase II (left) and γ -adaptin (right).

TGN, but there is no gross change in TGN morphology.

CBM Does Not Inhibit the Action of Diphtheria Toxin

To assess what other aspects of membrane traffic might be affected by CBM, we measured the effect of CBM on the sensitivity of CHO cells to diphtheria toxin. Diphtheria toxin kills cells by a mechanism that requires three major steps (Middlebrook and Dorland, 1984; Eidels and Draper, 1988). 1) Receptor-mediated endocytosis of the toxin bound to a cell surface receptor initially brings the toxin into the cell inside a vesicle. 2) Insertion of the toxin into the vesicle membrane and transfer of the catalytic fragment of the toxin across the membrane into the cytosol occur. The insertion is initiated by a conformational change induced upon exposure of the toxin to an acidic pH

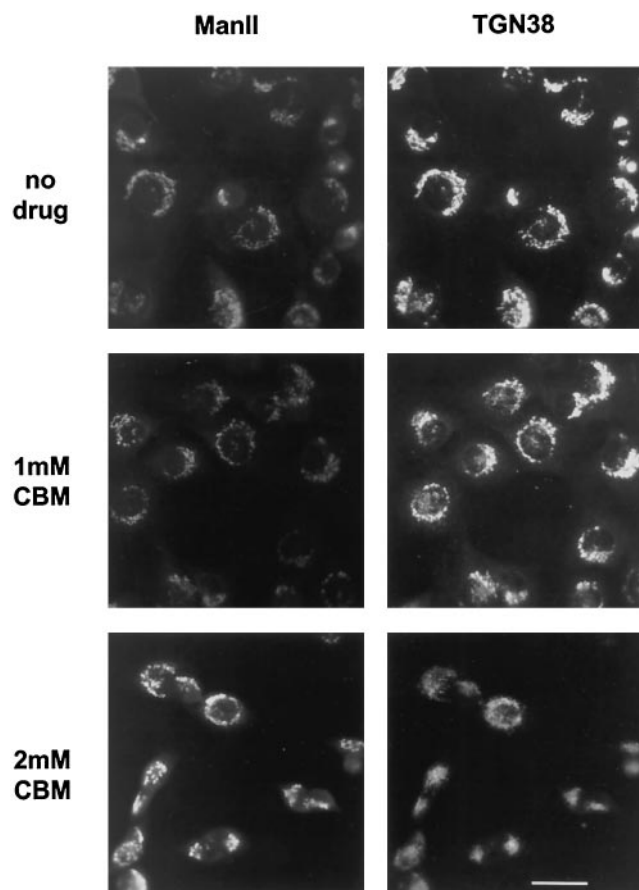


Figure 10. Distribution of mannosidase II and TGN38 in CBM-treated cells. NRK cells were prepared for immunofluorescence microscopy as described in Figure 4 except that the cells were double-labeled with antibodies to mannosidase II (left) and TGN38 (right).

within an endocytic vesicle. 3) The catalytic fragment inactivates elongation factor 2, arresting protein synthesis. The susceptibility of cells to diphtheria toxin is a sensitive indicator of the function of those aspects of membrane traffic required by the toxin for activity. The toxin is particularly sensitive to increases in vacuolar pH caused by lysosomotropic amines such as ammonium chloride. The data in Table 1 show that CBM had no consistent effect on the concentration of diphtheria toxin required to reduce protein synthesis by 50% (IC_{50}). This is evidence that CBM does not significantly impair receptor-mediated endocytosis of the toxin or the acidification of intracellular compartments.

DISCUSSION

We arrived at the study of CBM by its structural similarity to aminoglycoside antibiotics that are be-

Table 1. Effect of CBM on the sensitivity of CHO cells to diphtheria toxin

	CBM (mM)				
	0	0.1	0.5	1.0	1.5
IC ₅₀ (μg/ml) ^a	39	51	25	32	53

^a The IC₅₀ is the concentration of diphtheria toxin required to reduce protein synthesis by 50%. Each value is the average of three or more independent determinations at each CBM concentration.

lied to interact with the dilysine-binding sites on coatomer (Hudson and Draper, 1997). Like the aminoglycoside antibiotic Geneticin, as well as dilysine itself, CBM inhibited the binding of coatomer to Golgi membranes *in vitro*. CBM also strongly reduced the immunofluorescence signal of β -COP associated with the Golgi complex of intact cells, suggesting that CBM inhibits coatomer binding to Golgi membranes *in vivo* as well as *in vitro*. This inhibition is presumably mediated by the two strategically placed primary amino groups in CBM, similar to those in 2-deoxy-streptomycin, that appear to mimic the amino groups of dilysine. The simplest model for the effect of dibasic compounds on coating is that they occupy the dilysine-binding sites on coatomer and impair the binding of coatomer to membranes. *In vivo*, the steady-state extent of COPI association with membranes is likely a balance between coating and uncoating so that a block in the coating step eventually depletes Golgi membranes of COPI.

CBM inhibited transport to the cell surface of both influenza HA and the G protein of vesicular stomatitis virus, suggesting that the drug is a general inhibitor of secretion. The concentration of CBM that impaired secretion was in the range of 1–2 mM, similar to the concentration that reduced coatomer binding to the Golgi of intact cells. It is reasonable, therefore, that the block in coating could cause the block in secretion. Alternatively, CBM is a weak base and has the potential to raise the pH in acidic compartments such as the Golgi complex by ion trapping (de Duve *et al.*, 1974), which could conceivably cause COPI dissociation and block secretion. However, several lines of evidence argue that CBM does not interfere with secretion by altering proton gradients. First, at concentrations that impaired secretion, CBM did not appear to elevate the pH within acidic compartments. This is indicated by the observation that the cytotoxic activity of diphtheria toxin, which is very sensitive to the action of weak bases on pH (Middlebrook and Dorland, 1984; Eidels and Draper, 1988), was not affected by CBM. Second, intentionally elevating the pH inside acidic compartments with ammonium chloride did not alter the distribution of COPI on the Golgi complex of NRK cells.

Thus, even if CBM subtly raised the luminal pH of the Golgi compartment in a way undetected by diphtheria toxin, this alone would not affect COPI association with Golgi membranes. Third, 1,3-diaminopropane, a linear dibasic compound, did not impair secretion of HA, even at 10 mM (Figure 2). 1,3-Diaminopropane also had no effect on coatomer binding to Golgi membranes *in vitro* or *in vivo*. This further suggests that it is not simply the weakly basic nature of CBM that inhibits secretion; rather, a more specific feature of CBM is involved, probably the conformation of the amino groups constrained by the cyclohexane ring.

Previous work with brefeldin A, which dissociates COPI from Golgi membranes and causes the Golgi and ER membranes to fuse, led to the suggestion that the COPI coat was required for the structural integrity of the Golgi complex (Donaldson *et al.*, 1991b; Klausner *et al.*, 1992). We found, however, that the Golgi apparatus retained a recognizable morphology in the presence of CBM, even though the COPI normally bound to Golgi membranes was absent. Also, Golgi carbohydrate-modifying enzymes could not be biochemically detected within the ER after CBM treatment, indicating that CBM did not induce the Golgi and ER membranes to fuse. This is consistent with the maintenance of Golgi integrity in the presence of CBM and also suggests that dissociation of COPI is not sufficient in itself to induce the fusion of Golgi and ER membranes.

The mechanism of brefeldin A action is not well understood. However, an advance was made with the discovery that brefeldin A stimulates a mono-ADP-ribosylation activity that may underlie some effects of the drug on membrane traffic (De Matteis *et al.*, 1994; Di Girolamo *et al.*, 1995). This work has recently been extended to demonstrate that inhibiting the brefeldin A-dependent ADP-ribosylation activity results in dissociation of COPI from Golgi membranes upon brefeldin A addition, but the morphology of the Golgi complex is retained, and fusion with the ER does not occur (Mironov *et al.*, 1997; Weigert *et al.*, 1997). Our results with CBM are consistent with the work of Mironov *et al.* (1997) suggesting that dissociation of COPI from Golgi membranes is insufficient alone to cause a loss of Golgi complex morphology and fusion with the ER.

Three lines of evidence indicate that CBM does not inhibit secretion by impairing the transport of material from the ER to the Golgi. First, the acquisition of endoglycosidase H resistance by HA was only slightly delayed by CBM, indicating that HA was exposed to *N*-acetylglucosaminidase I and α -mannosidase II, enzymes necessary to process glycoproteins to endoglycosidase H resistance. Because CBM did not induce fusion of the Golgi with the ER, resistance to endoglycosidase H could not have resulted from the processing of HA by Golgi enzymes that had been returned to the ER. The fact that the Golgi and ER remained

separate under the influence of CBM also implies that dissociation of COPI did not cause massive unregulated membrane fusion. These data strongly suggest that HA was transported from the ER, through the intermediate compartment, to at least the cis/medial Golgi where the two processing enzymes are present. Second, upon shifting the temperature from 41 to 32°C in the presence of CBM, the G protein of VSV was transported out of the ER to a compartment that was identifiable as the Golgi complex by immunofluorescence microscopy. Third, when Golgi membranes were induced to fuse with the ER by brefeldin A, followed by removal of brefeldin A in the presence of CBM, a Golgi complex with a perinuclear morphology recognizable by immunofluorescence microscopy was reformed. Thus, at least α -mannosidase II, the antigen used in these immunofluorescence experiments, exited the ER to become part of a perinuclear Golgi complex in the presence of CBM. Other Golgi proteins presumably left the ER as well, although fine structural details of the reformed Golgi membranes are not available.

CBM strongly reduced the fluorescence signal from β -COP associated with Golgi membranes *in vivo*, even in the presence of AIF₄⁻, which normally stabilizes COPI binding to the Golgi complex. This suggests that CBM is very effective in preventing coatomer binding to the Golgi complex. However, the possibility cannot be dismissed that residual membrane-bound COPI could still be present and serve functions necessary for transport from the ER to the Golgi, although transport from the Golgi to the plasma membrane is blocked. This prospect is particularly important in light of evidence that anterograde transport by coatomer may be mediated by binding to diphenylalanine motifs (Fiedler *et al.*, 1996; Fiedler and Rothman, 1997), an interaction that might not be impaired by CBM. Nevertheless, our results suggest that a full complement of COPI on membranes is not essential for normal ER-to-Golgi transport in mammalian cells, which seems to differ from the interpretation of several lines of evidence in the literature. Microinjection into cells of an antibody to β -COP (anti-EAGE) inhibits by 50% the acquisition of resistance to endoglycosidase H by VSV G protein (Pepperkok *et al.*, 1993) and inhibits by 50% the reformation of the Golgi upon removal of brefeldin A (Scheel *et al.*, 1997). However, recent work indicates that anti-EAGE does not inhibit the binding of coatomer to membranes; rather, it inhibits the dissociation of COPI from membranes (Scheel *et al.*, 1997). Other agents that block COPI dissociation, GTP γ S (Scales *et al.*, 1997) and the GTP-restricted form of ARF1 (Aridor *et al.*, 1995), also impair transport from the intermediate compartment to the Golgi. These results suggest that the dissociation of COPI from certain membranes may be necessary for transport to the Golgi, but they do not seem to contradict the possibil-

ity that ER-to-Golgi transport can occur in the absence of bound COPI.

Balch and coworkers examined the role of COPI in transport from the ER to the Golgi with semi-intact cells (Peter *et al.*, 1993; Aridor *et al.*, 1995; Rowe *et al.*, 1996; Tisdale *et al.*, 1997) and concluded that COPI was necessary for the recycling of membrane from the intermediate compartment back to the ER but was not needed for vesicle formation from the ER. However, there were conflicting data on whether coatomer recruitment to membranes was directly needed for transport from the intermediate compartment to the Golgi. ARF1(T31N), the GDP-restricted form of ARF1 that blocks coatomer binding, inhibited ER-to-Golgi transport, suggesting that COPI recruitment was essential to reach the Golgi (Aridor *et al.*, 1995; Rowe *et al.*, 1996). In contrast, depleting cytosol of coatomer and ARF1 did not inhibit transport from the intermediate compartment to the Golgi, leaving open the question of whether coatomer binding was directly needed to complete transport to the Golgi (Rowe *et al.*, 1996). A role for COPI in ER-to-Golgi transport has also been inferred from studies of the temperature-sensitive ldlf mutant of CHO cells that are defective in ϵ -COP (Guo *et al.*, 1994; Scales *et al.*, 1997). However, not all consequences of the lesion are consistent with such a role. For example, the Golgi complex is disorganized within 6 h of placing ldlf cells at 40°C (Guo *et al.*, 1994), but at 7 h after the temperature shift, low density lipoprotein is still converted to an intermediate form that is resistant to digestion by endoglycosidase H, suggesting that it has reached the cis/medial Golgi (Hobbie *et al.*, 1994). In addition, ldlf cells do not exhibit a brefeldin A-like phenotype at the restrictive temperature despite the absence of ϵ -COP (Daro *et al.*, 1997). Several hours or more are required to induce the phenotype in ldlf cells, and it is difficult to separate primary defects in ER-to-Golgi transport from secondary consequences of the lesion. Altogether, there is no conclusive evidence that COPI is directly required for ER-to-Golgi transport in mammalian cells, and our results with CBM may reflect that COPI is not critical for this process.

CBM-induced dissociation of COPI from membranes correlated with defective transport from the Golgi to the plasma membrane, but it is not clear what transport event is blocked. The Golgi complex as seen by immunofluorescence microscopy appeared distended and swollen, which might be expected if membrane from the ER had entered the cis side of the Golgi but had not exited the trans side. Two popular models to explain the anterograde transport of material through the Golgi apparatus are the vesicular transport model and the maturation model. The vesicular transport model proposes that vesicles, presumed to use a COPI coat, carry material from one stack to the next in the trans direction. The maturation model pro-

poses that one stack matures into the succeeding stack without anterograde vesicular movement, but the maturation process does imply retrograde vesicular transport in the trans-to-cis direction by COPI-coated vesicles to recover material from succeeding stacks. Thus, the dissociation of COPI from Golgi membranes induced by CBM should have adverse effects on secretion regardless of the model.

An unexpected consequence of CBM treatment was reduced γ -adaptin binding to the TGN. It is not clear why CBM should impair γ -adaptin binding to membranes, but a common factor required for binding of both coatomer and γ -adaptin to membranes is ARF1. Conceivably, the depletion of COPI from Golgi membranes could trap the GTP-bound form of ARF1 on Golgi membranes in a futile attempt to restore COPI. This could exhaust the cytosolic pool of ARF1 and thereby interfere with γ -adaptin binding. Further work is needed to explore this issue.

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