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Retrograde transport of protein toxins under conditions of COPI dysfunction

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Abstract

Retrograde transport dependent on coat protein I (COPI) was impaired using two different approaches and the effects on the retrograde transport of protein toxins were investigated. One approach was to study ldlF cells that express a temperature-sensitive defect in the ε-COP subunit of COPI. The second approach was to treat cells with 1,3-cyclohexanebis(methylamine) (CBM), a drug that interferes with the binding of COPI to Golgi membranes. With both approaches, cells remained sensitive to a variety of protein toxins regardless of whether the toxins contained a KDEL motif. Moreover, cholera toxin, which contains a KDEL sequence, was observed by immunofluorescence microscopy to enter the endoplasmic reticulum of Vero cells in the presence of CBM. These data support published evidence indicating the presence in cells of a COPI- and KDEL receptor-independent pathway of retrograde transport from the Golgi complex to the endoplasmic reticulum. In addition, the results suggest that certain toxins containing a KDEL motif may use either the COPI-dependent or COPI-independent pathway of retrograde transport.

Keywords: Golgi; Retrograde transport; Coat protein I; Ricin; Cholera toxin

1. Introduction

Cholera toxin (CT), ricin, Shiga toxin (including Shiga-like toxin 1 or verotoxin) and *Pseudomonas aeruginosa* exotoxin A (ETA) are protein toxins that express their cytotoxic activities by catalytically modifying substrates in the cytoplasm of mammalian cells. Ricin and Shiga toxin arrest protein synthesis by depurinating 28S ribosomal RNA [1,2]. ETA arrests protein synthesis by transferring the ADP-ribosyl moiety of NAD^+ to elongation factor 2 [3]. CT ADP-ribosylates a regulatory G protein that results in activation of adenylate cyclase and elevation of cAMP [4]. To interact with cytoplasmic substrates, the catalytic subunits of the toxins must pass through a membrane and enter the cytoplasm, a process that initiates with receptor-mediated uptake of the toxins. The receptor for CT is the ganglioside G_{M1} [4] and the receptor for Shiga toxin is the glycolipid globotriaosylceramide [5]. Ricin binds to glycoconjugates containing galactose [6] and can potentially use either glycoproteins or glycolipids as a receptor. ETA interacts at the cell surface with the α_2-macroglobulin/low-density lipoprotein receptor-related protein [7]. After receptor binding at the plasma membrane and uptake into a vesicle, all four toxins are proposed to use retrograde transport through the Golgi complex to access the endoplasmic reticulum (ER)

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[8–13]. Upon entering the ER, the toxins are transported to the cytoplasm by a process that may involve the Sec61p complex [14].

The best-characterized pathway of retrograde transport through the Golgi complex is via vesicles coated with coat protein I (COPI). COPI is a macromolecular protein complex that envelopes vesicles budding from Golgi membranes [15–17]. The major structural component of COPI is coatomer, a protein complex comprising seven different polypeptides (α-, β-, β′-, γ-, δ-, ε-, and ζ-COP). Although a subset of COPI-coated vesicles may participate in anterograde transport through the Golgi stacks towards the plasma membrane [18–20], there is strong genetic and biochemical evidence that COPI-coated vesicles are involved in retrograde transport towards the ER [21,22]. Retrograde cargo includes luminal ER-resident proteins that have at their C-terminus the motif KDEL (single letter amino acid code) that specifies interaction with the KDEL receptor. Upon binding to the KDEL receptor in the Golgi complex, ER resident proteins that have escaped the ER are captured and returned to the ER via COPI-coated vesicles that contain the KDEL receptor. Some protein toxins, such as CT and ETA, bear a KDEL motif (or a closely related sequence) and it has been suggested that, once endocytosed, these toxins exploit the KDEL receptor and COPI-coated vesicles to reach the ER by retrograde transport [11,13].

It is intriguing that other protein toxins, such as Shiga toxin and ricin, do not contain a KDEL sequence and yet appear to use retrograde transport to enter the ER en route to the cytoplasm. In the absence of a KDEL motif, how do these toxins access a retrograde pathway? Recently, Girod et al. [23] presented direct evidence for the presence in cells of a COPI-independent retrograde pathway that is controlled by the small G protein Rab6 [24]. Shiga toxin appears to use this pathway to reach the cytoplasm, explaining why this toxin does not need a KDEL sequence [23] and the same pathway could also account for the KDEL-independent retrograde transport of ricin.

Within the framework of these recent advances in the understanding of retrograde membrane traffic and the action of protein toxins, those toxins with a KDEL sequence, such as CT, should use a COPI-dependent pathway to enter the ER. Therefore, disruption of COPI function should inhibit access of this type of toxin to the ER. In this paper we impaired COPI function in two different ways and studied the effects on the entry of protein toxins. In one approach, we used ldlF cells, which are a temperature-sensitive mutant of Chinese hamster ovary (CHO) cells that contain a single point mutation in the ε-COP subunit [25,26]. At the restrictive temperature, ldlF cells have a severely disorganized Golgi complex and are defective in secretion [25,27]. There is also a loss of ε-COP in ldlF cells to levels below detection within about 2 h after shifting to the restrictive temperature [26,28]. The second condition to disrupt COPI function was treating cells with 1,3-cyclohexanebis(methylamine) (CBM), a drug that appears to interact with coatomer and inhibits coatomer binding to Golgi membranes [29,30]. We measured the sensitivity of ldlF cells and cells treated with CBM to several toxins and found that they were sensitive to the toxins, including CT that contains the KDEL retrieval signal. In addition, CT was observed by immunofluorescence microscopy to enter the ER despite dysfunction of COPI-mediated retrograde transport. These data support evidence for the existence of a pathway to the ER that does not depend on COPI, but they also suggest that even a toxin that contains a KDEL sequence, such as CT, can use the COPI-independent retrograde pathway to reach the ER.

2. Materials and methods

2.1. Materials

Ricin, bovine serum albumin and secondary antibodies used with both rabbit and mouse primary antibodies were purchased from Sigma (St. Louis, MO, USA). CT was from Calbiochem (La Jolla, CA, USA). *P. aeruginosa* ETA was from Dr. S. Leppla (National Institute of Dental Research, National Institutes of Health, Bethesda, MD, USA). Rabbit antiserum to the CT A chain was from Drs. I. Majoul and H.-D. Söling (Universität Göttingen, Göttingen, Germany). Rabbit antiserum to mannosidase II (mann II) was purchased from Dr. K. Moremen (University of Georgia, Athens, GA, USA). Rabbit antiserum to TGN-38 was generously provided by

Dr. S. Milgram (University of North Carolina, Chapel Hill, NC, USA). Hybridoma cells producing monoclonal antibody M3A5 were originally from Dr. T. Kreis. Mouse monoclonal antibody to γ-adaptin was purchased from Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies to calnexin and monoclonal antibodies to calreticulin and the KDEL receptor were from StressGen Biotechnologies (Victoria, BC, Canada). Tran ^{35}S label was from ICN Radiochemical (Irvine, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was from Irvine Scientific (Santa Ana, CA, USA). Fetal bovine serum was from HyClone (Logan, UT, USA). CBM was from Acros Organics (Pittsburgh, PA, USA). Floromount G was from Fisher Scientific (Pittsburgh, PA, USA). cAMP was measured with a kit purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Cells and cell culture

LdlF cells, originally derived from CHO cells, were provided by Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA, USA). LdlF cells have a point mutation in ε-COP that makes them temperature-sensitive for growth [25–27]. We made a variant of ldlF cells that was not temperature-sensitive for growth, termed ldlF(ε-COP) cells, by transfecting ldlF cells with cDNA encoding wild-type human ε-COP. cDNA for human ε-COP was obtained from the IMAGE consortium (No. 40054) and was subcloned into the vector pCDNA3.1(Zeo) (Invitrogen Life Technologies, Carlsbad, CA, USA). LdlF cells at 34°C were transfected with purified plasmid DNA using LipofectAmine 2000 (Invitrogen Life Technologies) according to manufacturer's directions. The cells were then grown for 2 weeks at 34°C with 300 μg/ml Zeocin and the cells in 12 surviving colonies were subcloned and checked for growth at 39.5°C. Cells from one of the colonies that thrived at the high temperature were chosen for further use. Vero cells and NRK cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were routinely grown in DMEM supplemented with 10 mM HEPES and 5% fetal bovine serum in incubators with 90% air and 10% CO_2 at either 34°C, 37°C or 39.5°C as required.

2.3. Protein synthesis assays

Protein synthesis was measured by the incorporation of radioactivity from Tran ^{35}S label into acid-insoluble protein essentially as described previously [29]. Cells were pretreated as described in the figure legends and a toxin was added at different concentrations for the indicated times. Tran ^{35}S label (1 μCi/ml) was added for 30 min and the cells were washed, lysed, and the lysate spotted within squares defined by gridlines drawn on filter paper. The filter paper was incubated in 5% trichloroacetic acid containing 0.5 mg/ml methionine for 30 min at room temperature, washed twice for 5 min in 100% ethanol, and dried. Radioactivity within each square of the grid was measured with a PhosphorImager using volume quantitation (Molecular Dynamics, Sunnyvale, CA, USA). The IC_{50} value is defined as the concentration of toxin required to inhibit protein synthesis by 50% compared to controls that received no toxin.

2.4. Kinetics of protein synthesis inhibition by ricin

The rate of ricin intoxication was measured by assessing the incorporation of radioactivity from Tran ^{35}S label into acid-insoluble protein as described previously [31] with modifications. Cells were plated at 2×10^5 cells per well in four-well culture dishes 2 days before an experiment. After overnight incubation at 34°C, cells were shifted to 39.5°C for 18 h. The cells were chilled at 0°C and incubated with DMEM (lacking methionine) in the presence of ricin (100 μg/ml) for 1 h to allow toxin binding. Intoxication was initiated by replacing the cold medium with prewarmed assay medium containing the same concentration of toxin, and incubation was continued at 39.5°C. Tran ^{35}S label (10 μCi/ml) was added for 10 min at desired time points. The cells were washed, lysed and radioactivity measured as described in the preceding section. Radioactivity in samples not treated with toxin was taken as 100% protein synthesis.

2.5. Cholera toxin assays

The sensitivity of CHO and ldlF cells to CT was measured by shape changes induced in the cells upon

action of CT [32]. Cells were plated on 12 mm diameter glass coverslips at 34°C overnight followed by incubating half of the samples at 39.5°C for 6 h to induce the lesion in the mutant cells. The medium was replaced with DMEM containing the desired concentration of CT and samples were incubated at either 34°C or 39.5°C for 18 h. Cells were fixed in 4% paraformaldehyde and washed with phosphate buffered saline three times. Coverslips were mounted and images obtained with a Nikon TE300 microscope and a 40× apochromat lens. The width and the length of individual cells in two fields (each containing 10–20 cells) from each of two independent experiments were measured and the ratio of length to width was plotted as a function of CT concentration.

The effect of CT on cAMP levels in ldlF(ε-COP) and ldlF cells was also directly measured. Cells in 24-well culture plates were incubated at either 34°C or 39.5°C in DMEM containing 5% fetal bovine serum for times indicated in the legends and the desired concentration of CT was added. Incubation was continued for the times indicated in the figures and tables at either 34°C or 39.5°C and the level of intracellular cAMP was determined by immunological assay according to instructions supplied with the Amersham Pharmacia Biotech assay kit.

The effect of CBM on the activity of CT with Vero cells was also determined by measuring the levels of cAMP essentially as described in the previous paragraph. Vero cells cultured in 24-well plates were incubated for 45 min on ice with the indicated concentrations of CT to allow toxin binding. DMEM adjusted to pH 8.8 as previously described [29] was added at 37°C with or without 2 mM CBM. After 60 min, the cells were lysed and intracellular cAMP was measured according to instructions supplied with the kit.

2.6. Immunofluorescence microscopy

LdlF cells were plated on 12 mm diameter glass coverslips at 34°C 1 day before an experiment. Half of the samples were then shifted to 39.5°C and incubated as described in the figure legends. For indirect immunofluorescence, cells were fixed in 4% paraformaldehyde and permeabilized by 0.2% saponin for 10 min. Samples were then washed three times with phosphate buffered saline and incubated with phosphate buffered saline containing 1% BSA for 10 min. Primary antibodies were incubated with fixed and permeabilized cells for 30 min at room temperature, washed as in the previous sentence, followed by addition of secondary antibody and a final washing.

Vero cells were plated on glass coverslips 1 day before an experiment. The cells were washed twice with phosphate buffered saline to remove serum followed by incubation for 45 min on ice with 2 μg/ml CT in serum-free DMEM to allow toxin binding. The medium was removed and fresh DMEM adjusted to pH 8.8 with or without 2 mM CBM was added for times indicated in the figure legends. The cells were fixed with 4% paraformaldehyde for 10 min, washed three times with phosphate buffered saline and permeabilized with cold methanol for 15 min. The cells were rinsed three more times with phosphate buffered saline and incubated with phosphate buffered saline containing 1% bovine serum albumin. Incubations with primary and secondary antibodies were 30 min with washings between antibody applications as in the previous sentence.

Coverslips were mounted and viewed with a Nikon TE300 microscope equipped with epi-illuminated fluorescence and a 60× lens (NA = 1.4). Images were obtained with a MicroMax digital camera (Princeton Instruments, Trenton, NJ, USA). Pixel intensities were adjusted to full scale and figures were assembled in Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA, USA). Images were printed with a Codonics NP 1660 printer (Cleveland, OH, USA).

3. Results

3.1. The interaction of toxins with ldlF cells

LdlF cells were examined by immunofluorescence microscopy after staining with various Golgi membrane markers at permissive and restrictive temperatures to characterize aberrations in the Golgi apparatus caused by the ε-COP defect. Antibodies to the β subunit of coatomer were used as a marker for COPI. The cis/medial Golgi complex was identified by antibodies to α-mannosidase II (mann II), a transmembrane resident of the Golgi complex. Anti-γ-adaptin was used to stain the trans Golgi network (TGN). In ldlF cells at 34°C, all these antibod-

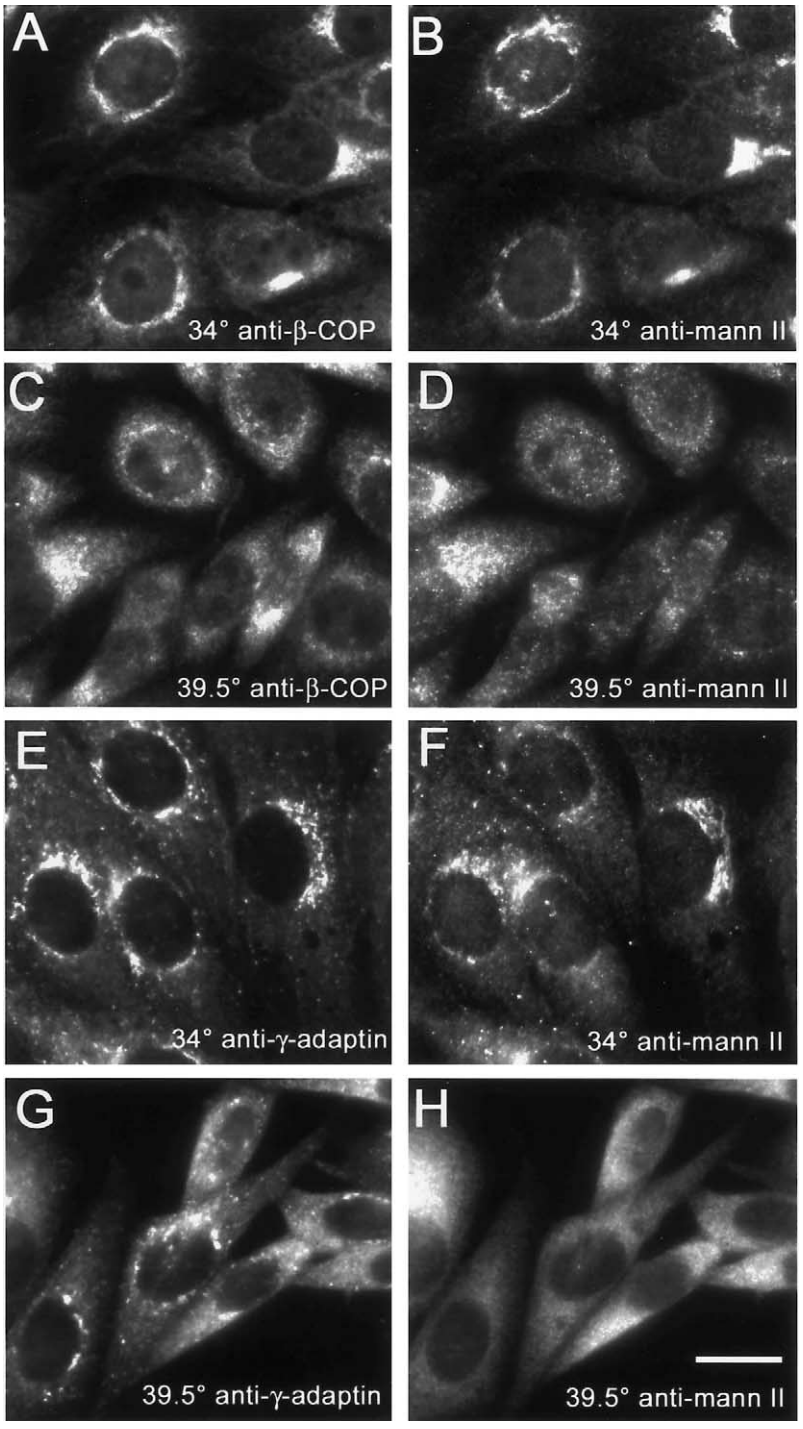

Fig. 1. The effect of the lesion in ldlF cells on the subcellular distribution of β-COP, mann II and γ-adaptin. Cells at 34°C (A,B,E,F) were fixed and stained for double immunofluorescence with primary antibodies to β-COP (A) and mann II (B) or to γ-adaptin (E) and mann II (F). Cells incubated at 39.5°C for 6 h (C,D,G,H) were fixed and stained for double immunofluorescence with primary antibodies to β-COP (C) and mann II (D) or γ-adaptin (G) and mann II (H). The bar in H is 10 μm.

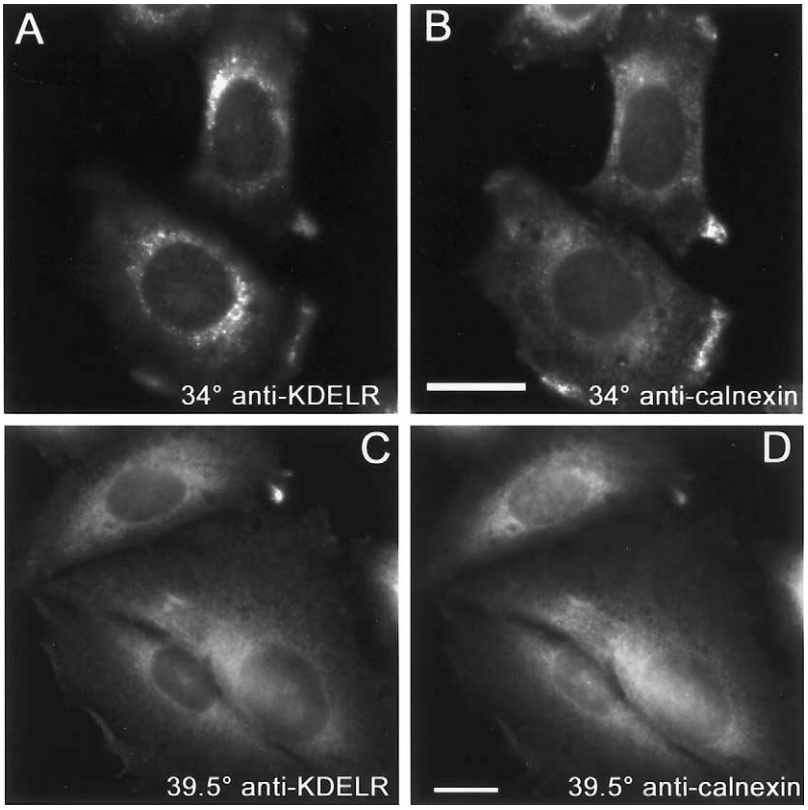

Fig. 2. The effect of the lesion in ldlF cells on the subcellular distribution of the KDEL receptor and calnexin. Cells at 34°C (A,B) were fixed and stained for double immunofluorescence with primary antibodies to the KDEL receptor (A) and calnexin (B). Cells incubated at 39.5°C for 6 h (C,D) were fixed and stained for double immunofluorescence with primary antibodies to the KDEL receptor (C) and calnexin (D). The bar in B (10 μm) is for panels A and B. The bar in D (10 μm) is for panels C and D.

ies co-localized in a typical Golgi-like pattern adjacent to the nucleus (Fig. 1A,B,E,F). After 6 h at 39.5°C (Fig. 1C,D), staining for β-COP and mann II became diffuse with small spots of fluorescence evident, similar to what has previously been observed in ldlF cells at the restrictive temperature [25,26,33]. Interestingly, anti-γ-adaptin continued to stain perinuclear membrane at the restrictive temperature while anti-mann II did not (Fig. 1G,H). In further experiments, the membrane that reacted with anti-γ-adaptin also co-localized with antibodies to TGN-38, suggesting that the TGN, or vestiges of the TGN, remained in ldlF cells at the high temperature (data not shown). Altogether, the results in Fig. 1 confirmed that ldlF cells have a disorganized Golgi complex at the restrictive temperature, although remnants of the TGN that do not stain for mann II appear to persist.

The fate of the KDEL receptor in the mutant cells was also investigated. At 34°C, the KDEL receptor was present in a perinuclear Golgi-like pattern and in a more diffuse distribution resembling the ER (Fig. 2A,B). After 6 h at 39.5°C, there was no evidence for association of the KDEL receptor with Golgi mem-

Table 1
Sensitivity of ldlF(ε-COP) and ldlF cells to ricin at 34 and 39.5°C

Cells	°C	IC_{50} (ng/ml ± S.E.M.)
ldlF(ε-COP)	34	81 ± 16
ldlF	34	15 ± 9
ldlF(ε-COP)	39.5	25 ± 0.4
ldlF	39.5	2.3 ± 0.01

Cells were incubated at 39.5°C for 14 h or maintained at 34°C and treated with different concentrations of ricin for 6.5 h. Tran ^{35}S was added for the last 30 min of the incubation and the incorporation of radioactivity into acid-insoluble material was determined as described in Section 2. Protein synthesis in ldlF cells was not impaired by incubation at the high temperature compared to the control cells. IC_{50} values are the average of three or four determinations ± S.E.M.

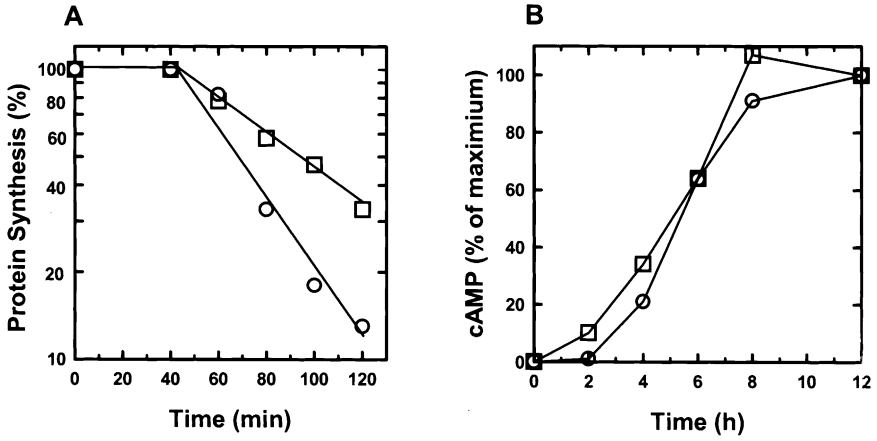

Fig. 3. The kinetics of ricin and CT action on control and ldlF cells at 39.5°C. (A) Rate of protein synthesis inhibition by ricin in control and ldlF cells at 39.5°C. Control (□) and ldlF (○) cells were shifted to 39.5°C for 18 h, chilled to 0°C, and incubated with 100 μg/ml ricin for 1 h to bind the toxin to cell surface receptors. The cells were raised to 39.5°C by addition of warm medium containing 100 μg/ml ricin and protein synthesis was assessed at the indicated times as described in Section 2. (B) LdlF (ε-COP) control cells (□) and ldlF (○) cells were shifted to 39.5°C for 4 h and incubated with 1 μg/ml CT. At the times indicated, the cellular amount of cAMP was determined as described in Section 2.

branes of ldlF cells (Fig. 2C,D); instead, the receptor appeared to distribute in a diffuse pattern similar to that for the ER marker calnexin. The absence of COPI on perinuclear Golgi membranes, coupled with the observation that the KDEL receptor is apparently confined to the ER, suggests that COPI-dependent retrograde transport does not function in the mutant cells at high temperature.

The sensitivity of control cells and ldlF cells to ricin at permissive and restrictive temperatures is shown in Table 1. The concentration of ricin that reduced protein synthesis by 50% (IC_{50}) in ldlF(ε-COP) control cells (a derivative of ldlF cells that expresses wild-type ε-COP) was 81 ng/ml at 34°C and 25 ng/ml at 39.5°C, an increase in sensitivity of 3.2-fold in response to the high temperature. The IC_{50} values for ricin with ldlF cells at 34°C and 39.5°C were 15 and 2.3 ng/ml, respectively, an increase in sensitivity of 6.5-fold at the high temperature. Thus, inducing Golgi dysfunction at the high temperature did not inhibit the action of ricin compared to control cells; rather, the sensitivity of ldlF cells to the toxin was slightly enhanced. It is not clear why ldlF cells are approx. 5–10 times more sensitive to ricin than control cells at either the permissive or restrictive temperatures, but it may be related to the fact that the level of ε-COP is about half of normal in the mutant cells even at 34°C [26]. Thus, a mild Golgi defect may manifest itself even at 34°C that slightly sensitizes the cells to ricin.

To characterize the entry of ricin into ldlF cells with greater precision than an IC_{50} assay, we measured the time required for ricin to be transported from the cell surface to the cytoplasm. Cells were preincubated at the restrictive temperature for 18 h, quickly chilled and incubated with saturating concentrations of ricin to allow occupation of cell surface receptors. The cells were returned to the restrictive temperature and protein synthesis was assessed at different times. A plot of the logarithm of the inhibition of protein synthesis versus time yields a straight line in this type of analysis, and extrapolation of the graph to no inhibition of protein synthesis provides the time required for the first burst of toxin molecules to reach the cytoplasm [34]. There was no significant difference in the time (40–45 min) at which ricin first reached the cytoplasm of control and ldlF cells at the high temperature (Fig. 3A). After nearly identical lag periods, the rate of intoxication for ldlF cells was slightly greater than that for control cells, most likely related to the fact that ldlF cells are more sensitive to ricin than control cells at the restrictive temperature. This result indicates that the absence of ε-COP in the mutant cells does not limit the rate at which ricin is transported from the cell surface to the cytoplasm.

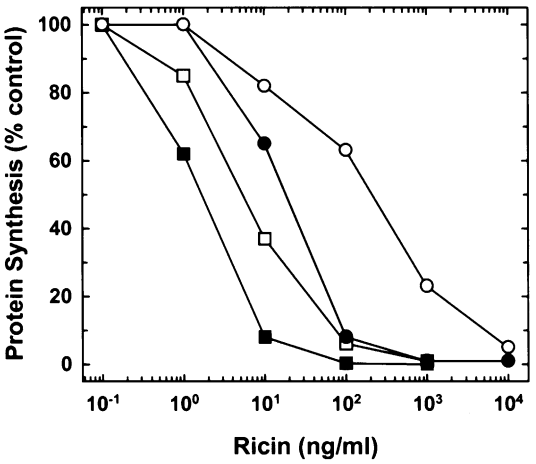

Fig. 4. The effect of brefeldin A on the cytotoxic activity of ricin at the restrictive temperature with ldlF cells and control cells. Cells were incubated at 39.5°C for 14 h and treated with different concentrations of ricin for 6.5 h. Brefeldin A (5 μg/ml) was added to some cells 30 min before the addition of ricin. Tran ^{35}S was added for the last 30 min of the incubation and the incorporation of radioactivity into acid-insoluble material was determined as described in Section 2. ■, ldlF cells without brefeldin A; □, ldlF cells with brefeldin A; ●, ldlF(ε-COP) cells without brefeldin A; ○, ldlF(ε-COP) cells with brefeldin A.

The sensitivity of ldlF cells to ricin at the restrictive temperature despite dysfunction of the Golgi complex raises the question of whether the mechanism of toxin entry into the cytoplasm of the mutant cells at high temperature is the same as in normal cells. If the same mechanism is used for both normal and mutant cells, then an inhibitor of toxin entry into normal cells should also inhibit toxin entry with ldlF cells. Brefeldin A normally inhibits the cytotoxicity of ricin [35] and we compared the effect of brefeldin A on the action of ricin with ldlF and ldlF(ε-COP) control cells at the restrictive temperature. Both ldlF and ldlF(ε-COP) cells incubated at 39.5°C required a higher concentration of ricin to reduce protein synthesis in the presence of brefeldin A (Fig. 4). This suggests that a brefeldin A-sensitive step is required for ricin to reach the cytoplasm in both mutant and normal cells.

Ricin does not contain a KDEL signal and the sensitivity of ldlF cells to this toxin at high temperature despite the COPI defect can be explained if ricin uses the COPI-independent pathway of retrograde transport to reach the ER and hence the cytoplasm [23,24]. In contrast to ricin, a toxin such as CT that bears a KDEL signal is predicted to require the COPI-dependent pathway of retrograde transport to reach the ER en route to the cytoplasm [23,24]. It was of interest therefore to determine whether ldlF cells were sensitive to CT and two different assays were used in this determination. In the first, we exploited the observation that the elevation of cytoplasmic cAMP caused by CT changes the shape of CHO cells from a rounded morphology to an elongated spindle-shaped morphology and that the sensitivity of CHO cells to CT can be assessed by measuring the ratio of cell length to width [32]. The elongation of mutant and CHO cells at both restrictive and permissive temperatures as a function of CT concentration was about the same, indicating that ldlF cells retained sensitivity to CT at the restrictive temperature (Fig. 5).

The second assay for sensitivity to CT was to directly measure the effect of CT on cAMP levels in ldlF and ldlF(ε-COP) control cells at 34°C and 39.5°C (Table 2). At 34°C, cAMP in mutant and control cells was elevated over 120-fold by CT. At 39.5°C, cAMP was elevated about 120-fold in control cells and 60-fold in ldlF cells. This indicates that ldlF cells are sensitive to CT at the high temperature by this assay, although the increase in cAMP is not as great as in control cells. The difference between the morphological and cAMP assays with ldlF cells suggests that the morphological change is induced at a level of cytoplasmic cAMP that is below the max-

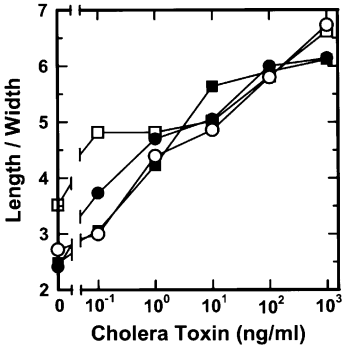

Fig. 5. The sensitivity of CHO and ldlF cells to CT at permissive and restrictive temperatures. Cells were plated on glass coverslips at 34°C for 1 day followed by incubation at either 34°C or 39.5°C for 6 h. The medium was replaced with DMEM containing the indicated concentrations of CT and incubation was continued at either 34°C or 39.5°C for 18 h. Cells were fixed and the length-to-width ratios were determined as described in Section 2. ■, CHO cells at 34°C; □, CHO cells at 39.5°C; ●, ldlF cells at 34°C; ○, ldlF cells at 39.5°C.

Table 2
The effect of CT on cAMP in ldlF(ε-COP) and ldlF cells at permissive and restrictive temperatures

Cells	°C	No CT	cAMP (fmoles/10^4 cells ± S.E.M.)	
			1 μg/ml CT	1 μg/ml CT+ brefeldin A
ldlF(ε-COP)	34	< 0.1	13.7 ± 1.4	
ldlF	34	< 0.1	12.4 ± 0.4	
ldlF(ε-COP)	39.5	< 0.1	12.1 ± 1.5	< 0.1
ldlF	39.5	< 0.1	6.2 ± 0.5	< 0.1

imum elevation caused by the toxin. Thus, the full morphological response is seen with ldlF cells although the increase in cAMP is less than with control cells. We also measured the effect of brefeldin A on the action of CT and found that the drug abolished the elevation of cAMP caused by the toxin with both mutant and control cells at the high temperature (Table 2). This indicates that a brefeldin A-sensitive event is involved in the action of CT on ldlF cells, as with ricin, and further supports the idea that the pathway by which toxins reach the cytoplasm is similar in mutant and control cells at high temperature.

To compare the rate at which CT increased cAMP in mutant and control cells, cells were incubated at the restrictive temperature to induce the defect in the mutant cells, treated with CT, and the level of cAMP was measured at different times up to 12 h. At 2 and 4 h after CT internalization was initiated, there appeared to be a mild delay of about 30 min in the increase of cAMP in the mutant cells, although the maximum amount of cAMP was attained by both mutant and control cells between 8 and 12 h (Fig. 3B). These data suggest that the transport of CT from the plasma membrane to the cytoplasm may be less efficient when COPI is impaired in the mutant cells at the high temperature, but the delay is not dramatic.

We attempted in further experiments to correlate the entry of the CT A chain into the cytoplasm of ldlF cells at 39.5°C with morphological observation of the A chain entering the ER of the cells by immunofluorescence microscopy, as has been done with other cell lines [9,10]. However, the CT A chain could not be reliably detected within the ER of either control cells or ldlF cells at any temperature, indicating that CHO cells were not a suitable model system for monitoring the intracellular location of CT by immunofluorescence microscopy. Therefore, we turned our attention to analysis of toxin entry into other cells under conditions of Golgi dysfunction in the presence of CBM where the cytotoxic activity of toxins could be correlated with morphological observations.

3.2. The interaction of toxins with CBM-treated cells

CBM inhibits coatomer binding to Golgi membranes in vitro [30] and interferes with coatomer binding to the Golgi complex of intact NRK cells and Vero cells [29,36]. In the presence of CBM, NRK cells did not acquire resistance to ricin; rather, the drug slightly sensitized the cells to the toxin (Table 3). We also tested the sensitivity of NRK cells to ETA, which has a KDEL-like sequence, and found that CBM also sensitized cells to this toxin (Table 3). To extend this study to CT, the effect of CBM on the entry of CT into the cytoplasm of Vero cells was assessed by changes in the cellular levels of cAMP in the presence and absence of CBM. As seen in Table 4, CBM did not inhibit the ability of CT to elevate cAMP levels in Vero cells. Thus, CBM did not seriously impair the entry of toxins into the cy-

Table 3
The effect of CBM on the sensitivity of NRK cells to ricin and ETA

Toxin	IC_{50} ± S.E.M. (μg/ml)			
	No CBM	0.5 mM CBM	1.0 mM CBM	1.5 mM CBM
Ricin	10 ± 0.3	5 ± 2	1 ± 0.1	2 ± 1
ETA	88 ± 5	38 ± 5	18 ± 8	7 ± 1

Cells were incubated with the indicated concentrations of CBM for 30 min at 37°C. Ricin or ETA was added at different concentrations for 90 min and protein synthesis was measured as described in Section 2. IC_{50} values were determined from the dose–response curves and each value is the mean of three independent determinations ± S.E.M.

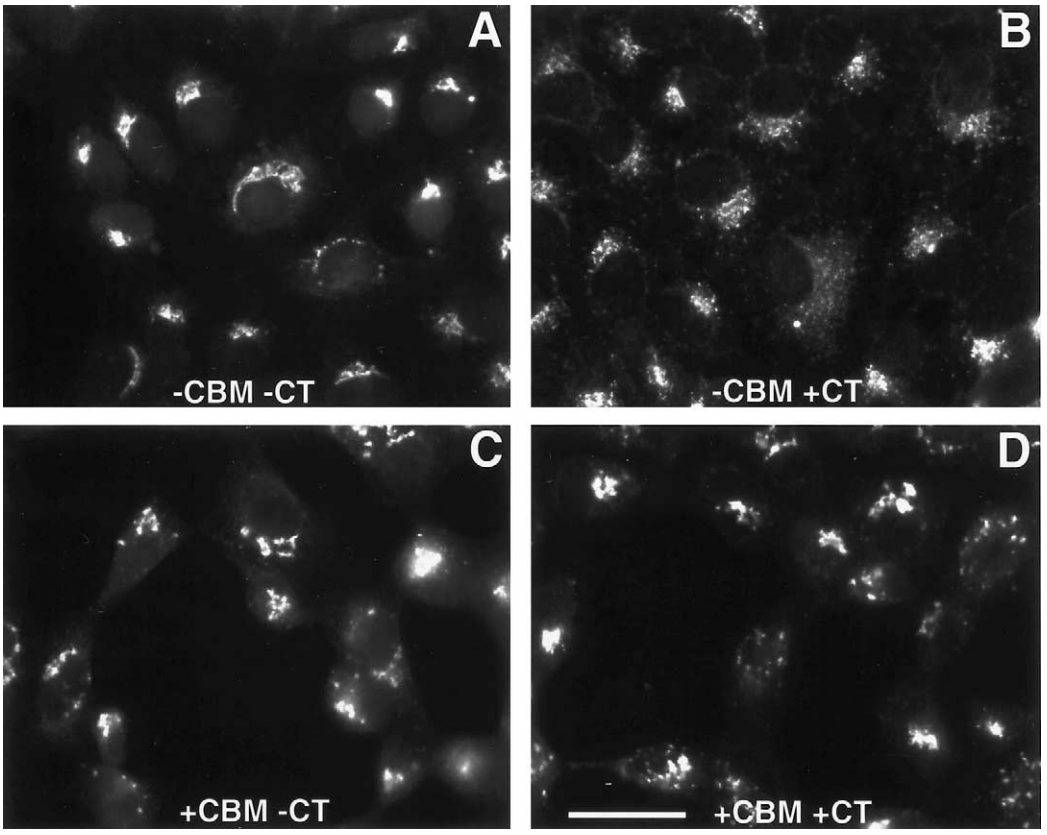

Fig. 6. The effect of CT on the distribution of the KDEL receptor in the presence and absence of CBM. Vero cells were incubated on ice for 45 min without CT (A,C) or with 2 μg/ml CT (B,D). Medium at pH 8.8 without CBM (A,B) or with 2 mM CBM (C,D) was added at 37°C for 90 min. The cells were fixed, incubated with antibodies to the KDEL receptor, and prepared for immunofluorescence microscopy as described in Section 2. The bar is 25 μm.

toplasm, even toxins such as CT and ETA that contain the KDEL motif.

Challenging Vero cells with CT normally causes a greater proportion of the KDEL receptor to redistribute into the ER by retrograde transport [10]. To verify that CBM impaired KDEL receptor-mediated retrograde transport, the influence of CT on the distribution of the KDEL receptor was measured with and without CBM. Vero cells were incubated with CT for 90 min in the presence or absence of CBM, fixed, and the location of the KDEL receptor was assessed. In the absence of CBM and CT, the KDEL receptor was in a ribbon-like perinuclear Golgi pattern typical for Vero cells (Fig. 6A). The addition of CT caused the KDEL receptor to assume a more punctate and spread pattern (Fig. 6B), reflecting redistribution of the receptor from the Golgi to the ER as a consequence of engaging the receptor in retrograde transport of CT [10]. In cells treated with CBM, the Golgi complex appeared swollen, a feature previously noted with other cells [29], and the KDEL receptor was present in Golgi membranes (Fig. 6C). The addition of CT did not cause any apparent redistribution of the KDEL receptor towards the ER in CBM treated cells (Fig. 6D). To quantitate the effect of CT on the distribution of the KDEL receptor in the presence and absence of CBM, the number of cells in several different fields either displaying a compact or disbursed distribution of the receptor

Table 4
The effect of CT on cAMP levels in the presence and absence of CBM with Vero cells

CBM	cAMP (fmoles/well)		
	No CT	0.1 μg/ml CT	1 μg/ml CT
−	342	3120	3090
+	396	2810	3050

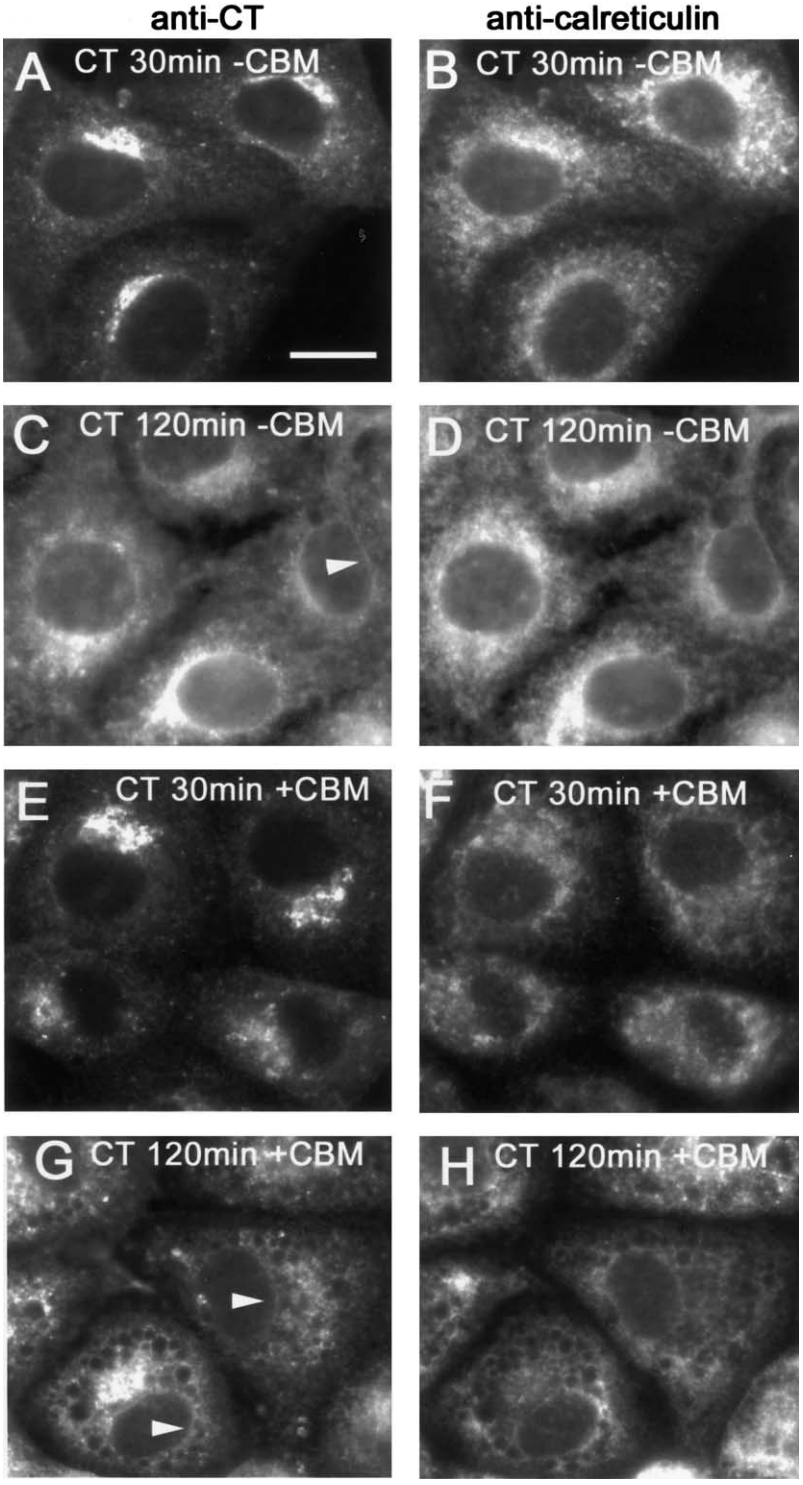

Fig. 7. The effect of CBM on the transport of CT. Vero cells were incubated on ice for 45 min with CT (2 μg/ml) to allow toxin binding. Medium at pH 8.8 without CBM (A–D) or with 2 mM CBM (E–H) was added at 37°C either for 30 min (A,B,E,F) or for 120 min (C,D,G,H). The cells were fixed and prepared for double immunofluorescence by staining with antibodies to CT (A,C,E,G) or antibodies to calreticulin (B,D,F,H). The arrowheads point to staining in the nuclear envelope. The bar is 10 μm.

Table 5
Quantitative analysis of the effect of CT on the distribution of the KDEL receptor in the presence and absence of CBM

Number of cells	CT	CBM	Compact (%)	Dispersed (%)
38	−	−	84	16
57	+	−	14	86
49	−	+	75	25
74	+	+	68	32

under different conditions were counted. As seen in Table 5, the addition of CT to cells in the absence of CBM resulted in an obvious redistribution of the KDEL receptor from a compact to a dispersed appearance. In the presence of CBM, this redistribution was strongly inhibited. These data argue that the retrograde transport of CT by a COPI and KDEL receptor-mediated process is indeed inhibited by CBM, consistent with the known effects of CBM on impairing COPI association with Golgi membranes.

To directly assess the effect of CBM on the entry of CT into the ER, cells were exposed to CT with or without CBM for 30 or 120 min and double-stained for the toxin and for the ER marker calreticulin (Fig. 7). In the absence of CBM, CT appeared at 30 min in a perinuclear pattern resembling the Golgi complex and at 120 min CT was within the ER, as indicated by diffuse staining similar to that for calreticulin and especially by staining of the nuclear envelope. In the presence of CBM, CT was again associated with a Golgi-like stain after 30 min and was evident within the nuclear envelope by 120 min. These data support the conclusion that CBM inhibits neither the elevation of cAMP caused by CT nor the transport of CT to the ER.

4. Discussion

We analyzed in this paper the interaction of toxins with cells in two different model systems that should disrupt retrograde transport dependent on COPI and the KDEL receptor. In ldlF cells, which lack the ε-COP subunit at the restrictive temperature, β-COP and mann II were distributed throughout the cytoplasm, often in punctate structures, and not present in a perinuclear location typical for the Golgi complex. This is similar to what has been reported by others who have studied ldlF cells by immunofluorescence and electron microscopy [25,27,33] and verifies that the Golgi apparatus is severely disrupted upon incubating the mutant cells at the restrictive temperature. We also noted that the KDEL receptor was not present in a Golgi-like morphology in ldlF cells at high temperature; rather, it appeared to be in the ER, suggesting it was not available to participate in retrograde transport. In the second system, cells were treated with CBM, a drug that interferes with the binding of COPI to Golgi membranes [29,30,36] and which should disrupt COPI-dependent retrograde transport. To confirm the disruption of retrograde transport, the effect of CBM on the CT-induced redistribution of the KDEL receptor from Golgi membranes to the ER was measured. In the absence of CBM, CT induced an obvious redistribution of the KDEL receptor away from the Golgi complex that is diagnostic for COPI-mediated retrograde transport. This redistribution was not evident in cells treated with CBM. These observations provide confidence that retrograde transport mediated by COPI and the KDEL receptor are seriously impaired in ldlF cells at high temperature and in cells treated with CBM.

When control cells were shifted from the permissive to the restrictive temperature, their sensitivity to ricin increased about 3-fold. The sensitivity of ldlF cells to ricin was increased 6-fold after the temperature shift. Thus, impairing COPI function at the high temperature did not cause resistance to ricin; rather, it slightly sensitized the cells to this toxin. There was also no significant difference between mutant and control cells in the rate at which ricin first began to inhibit protein synthesis at the high temperature, evidence that the defect in COPI had no effect on the rate of ricin transport from the cell surface to the cytoplasm. One potential explanation for the sensitivity of ldlF cells to ricin at the restrictive temperature is that a new and distinct pathway for reaching the cytoplasm independent of COPI is induced by high temperature only in the mutant cells. Two observations argue against this explanation. First, brefeldin A inhibited the effect of ricin on both control cells and ldlF cells at the high temperature. Brefeldin A disables ARF guanine nucleotide exchange factors

[37], suggesting that the action of ricin on both control and ldlF cells depends on a brefeldin A-sensitive exchange factor, consistent with the idea that ricin uses a similar pathway to enter both cell types. Second, the time required for ricin transport from the cell surface to the cytoplasm was the same for mutant and control cells at the restrictive temperature. This interval reflects the accumulated time of the various events involved in the transport process and the fact that the interval is the same for mutant and control cells is consistent with the idea that the transport process is the same. A more likely explanation for why ldlF cells remain sensitive to ricin at the high temperature is that they exploit a COPI-independent pathway normally present in cells to reach the cytoplasm.

The sensitivity of ldlF cells to CT at the high temperature was the same as that of control cells in the morphological assay. In the direct assay for the effect of CT on cytoplasmic cAMP, the level of cAMP in ldlF cells was increased about 60-fold by CT at the high temperature, half the increase seen with control cells. The rate at which CT increased cAMP in the mutant cells was mildly delayed compared to control cells at early time points, but the maximum increase was observed about 8 h after toxin addition for both mutant and control cells. This argues that the ability of CT to affect cAMP in the mutant cells at the high temperature in longer assays is not due to a few CT molecules that enter the cytoplasm of the mutant cells very slowly compared to the control cells. It is not clear why cAMP in the mutant cells was not increased to the same level as seen in control cells. One explanation is that two populations of mutant cells are present, one that responds fully to CT and the other that does not respond, resulting in an intermediate increase in the level of cAMP. However, the morphological assay, which measures the shape of individual cells, indicates that almost all cells in the mutant population respond to CT by changing shape. Thus, it is not likely that there are non-responding cells in the population. Although it is uncertain why CT does not increase cAMP levels in ldlF cells as much as in control cells, it is clear that ldlF cells retain significant sensitivity to CT despite the disruption in COPI.

Treating NRK cells with CBM to impair COPI function mildly enhanced sensitivity to ricin and ETA. The reason for this is unknown, but may stem from defects in secretion that occur when COPI is impaired, which should in turn impair lysosome biogenesis. Impaired lysosome biogenesis might lead to less effective degradation of toxins and consequently enhance cytotoxicity by increasing the lifetime of active toxins in the cell. It is known, for example, that impairing lysosome activity with drugs sensitizes cells to a variety of protein toxins [38,39]. CBM did not inhibit the ability of CT to elevate cAMP levels, nor did CBM block the transport of CT to the ER, as indicated in immunofluorescence studies. Although the conclusions based on work with CBM are subject to the caveat that there could be nonspecific effects with this drug that are not apparent, the results suggest that functional COPI is not essential for certain toxins to reach the ER, including toxins that bear a KDEL signal.

The model that has emerged for the trafficking of protein toxins that enter the ER en route to the cytoplasm is that toxins lacking a KDEL signal use the recently discovered COPI-independent pathway of retrograde transport, while toxins that bear a KDEL signal engage the KDEL receptor and use the COPI-dependent pathway [23,24,40]. The data presented here for ricin, which has no KDEL signal, support the existence of a COPI-independent pathway because ricin reached the cytoplasm even after COPI function was disrupted in two different ways. However, neither method of inhibiting COPI blocked the transport of CT to the cytoplasm, a surprising result because CT bears a KDEL signal. Moreover, CBM did not inhibit the transport of ETA to the cytoplasm and ETA also bears a KDEL-like signal. These results suggest that CT and ETA, and possibly other toxins that have a KDEL signal, can nevertheless engage the COPI-independent pathway and access the ER by a pathway that does not involve interaction of the KDEL motif with the KDEL receptor. Thus, there may be two pathways by which a toxin bearing a KDEL sequence can enter the ER and when one pathway is disrupted, the alternative is available.

If a toxin containing a KDEL motif can reach the ER by two pathways, one that requires the KDEL signal and another that does not, then the presence of the KDEL signal would not seem essential to target a toxin to the ER, raising the question of

what is the function of the KDEL signal present on some toxins. In addition to targeting toxins to the COPI-dependent pathway, an additional function of the KDEL signal could be that it contributes to cytotoxicity by facilitating retrieval back to the ER of toxin molecules that have entered the ER and then escaped, regardless of how the toxins initially entered the ER. Retrieval would presumably help maintain a higher concentration of toxin molecules in the ER, thus enhancing cytotoxicity. A role for the KDEL motif in retrieving toxins back to the ER is consistent with most of the data on toxin entry. Microinjected antibodies to the KDEL receptor and β-COP cause toxins that bear the KDEL signal to accumulate in the Golgi complex [23,41,42]. This accumulation would be predicted regardless of how the toxin entered the Golgi, either from endosomes in a retrograde direction or from the ER in an anterograde direction for toxins that had already entered the ER by a COPI-independent pathway. In both instances, KDEL-bearing ligands would bind the KDEL receptor trapped within Golgi cisternae and become trapped there themselves. The redistribution of the KDEL receptor from the Golgi complex towards the ER in the presence of CT [10] and ETA [42] is also compatible with the retrieval model as redistribution of the KDEL receptor from the Golgi to the ER was originally discovered for ligands coming from the ER [43].

If binding of the KDEL motif at the C-terminal end of a toxin to the KDEL receptor were essential to engage a pathway of membrane traffic that delivered the toxin to the ER, then removal of the KDEL signal from the toxin should strongly impair cytotoxic activity. However, removal of the KDEL sequence from CT reduces, but does not strongly block, CT activity [13]. Moreover, removing the KDEL-like sequence from *Escherichia coli* heat-labile toxin, which is very similar to CT, has little effect on activity [44]. The mild inhibition of cytotoxicity caused by removal of the KDEL motif from these toxins is consistent with the idea that there are two pathways a toxin may take to the ER: toxin transport still occurs when one pathway is unavailable, but less efficiently than when both are active. Attenuation of cytotoxicity when one pathway of retrograde transport is inhibited may also underlie our observations that the action of CT is mildly delayed in ldlF cells at high temperature and that CT does not elevate cAMP as much in ldlF cells at high temperature as in control cells.

Although removal of the KDEL sequence from CT mildly reduces cytotoxicity, removal of the KDEL-like motif (REDLK) from ETA strongly impairs cytotoxicity [11]. This seems to argue that engaging the COPI-dependent pathway is critical to deliver ETA to the ER. However, it is difficult to judge the significance of this argument because ETA has more complicated requirements for intoxication than some other toxins. For example, ETA containing the normal REDLK sequence fails to bind the KDEL receptor in vitro [45]. The terminal lysine residue can be removed by a protease present in plasma to generate a form of ETA that does bind the KDEL receptor, but the affinity of the interaction is lower than would be expected for efficient intoxication [46]. Thus, ETA may not be a good paradigm for assessing the effects of KDEL-like sequences on a toxin.

In summary, the results presented here are consistent with the following model for the retrograde transport of toxins to the ER. The toxins are internalized by receptor-mediated endocytosis and transported to the TGN. Two pathways lead from the TGN to the ER, one via retrograde transport through the Golgi cisternae that uses COPI and the KDEL receptor, and a distinct COPI-independent pathway. Toxins that have no KDEL sequence are channeled to the COPI-independent pathway and then to the ER. Certain toxins that contain a KDEL signal, such as CT, can exploit either the COPI-dependent or the COPI-independent pathway to reach the ER. Once within the ER, toxins that bear a KDEL signal and which escape the ER by entering the anterograde secretory pathway to the Golgi complex are retrieved back to the ER by the same COPI-dependent and KDEL receptor-dependent pathway that retrieves soluble resident ER proteins.

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