

# Transport of an External Lys-Asp-Glu-Leu (KDEL) Protein from the Plasma Membrane to the Endoplasmic Reticulum: Studies with Cholera Toxin in Vero Cells

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**Abstract.** The A2 chain of cholera toxin (CTX) contains a COOH-terminal Lys-Asp-Glu-Leu (KDEL) sequence. We have, therefore, analyzed by immunofluorescence and by subcellular fractionation in Vero cells whether CTX can be used to demonstrate a retrograde transport of KDEL proteins from the Golgi to the ER. Immunofluorescence studies reveal that after a pulse treatment with CTX, the CTX-A and B subunits (CTX-A and CTX-B) reach Golgi-like structures after 15–20 min (maximum after 30 min). Between 30 and 90 min, CTX-A (but not CTX-B) appear in the intermediate compartment and in the ER, whereas the CTX-B are translocated to the lysosomes. Subcellular fractionation studies confirm these results: after CTX uptake for 15

min, CTX-A is associated only with endosomal and Golgi compartments. After 30 min, a small amount of CTX-A appears in the ER in a trypsin-resistant form, and after 60 min, a significant amount appears. CTX-A seems to be transported mainly in its oxidized form (CTX-A1-S-S-CTX-A2) from the Golgi to the ER, where it becomes slowly reduced to form free CTX A1 and CTX-A2, as indicated by experiments in which cells were homogenized 30 and 90 min after the onset of CTX uptake in the presence of *N*-ethylmaleimide. Nocodazol applied after accumulation of CTX in the Golgi inhibits the appearance of CTX-A in the ER and delays the increase of 3', 5'cAMP, indicating the participation of microtubules in the retrograde Golgi-ER transport.

SOON after the detection of the COOH-terminal KDEL sequence in resident soluble proteins of the ER as a retention signal (Munro and Pelham, 1986), it became evident that the KDEL sequence is a retrieval rather than a retention signal (Munro and Pelham, 1987; Pelham, 1989). This view was mainly based on the observation that expression of the lysosomal enzyme cathepsin D with a COOH-terminal KDEL sequence led to the retention of most of the chimeric protein in the ER, but the glycan structure of the protein indicated that it had traversed the *cis*- and medium-Golgi cisternae before returning to the ER. Immunofluorescence (Lewis and Pelham, 1992; Hsu et al., 1992; Tang et al., 1993; Sönnichsen et al., 1994) and immunoelectronmicroscopical studies (Tang et al., 1993; Griffiths et al., 1994) showed that under steady-state conditions, the KDEL receptor ERD2 exists mainly in Golgi-like structures, particularly in the *cis*-Golgi network. The affinity of the KDEL receptor for the KDEL motif is increased under the slightly acidic conditions prevailing in the Golgi cisternae and is decreased under the neutral pH conditions in the ER (Wilson et al., 1993).

From these observations, it was deduced that: KDEL proteins can escape from the ER, they are "picked up" by the KDEL receptor, they are transported back to the ER together with the receptor, and are finally released in the ER. Up to now, however, the manner in which the occupied KDEL receptor is recycled to the ER remained unclear. Brefeldin A (Lippincott-Schwartz et al., 1990) as well as overexpression of the KDEL receptor (Hsu et al., 1992; Townsley et al., 1993) led to a change in the steady-state distribution of the KDEL receptor in favor of the ER. The overexpression of the secretory protein lysozyme fused to a COOH-terminal KDEL signal also resulted in a shift in the steady-state distribution of the KDEL receptor to the ER. None of these conditions is physiological as indicated, e.g., by the fact that under these conditions, not only the KDEL receptor, but also the Golgi markers mannosidase II and UDP-galactosyl transferase, were translocated from the Golgi to the ER.

A possible alternative method would be to channel a KDEL protein from the outside of the cell into the Golgi, where it might interact with the KDEL receptor and subsequently become transported to the intermediate compartment and to the ER. Recently, Miesenböck and Rothman (1995) have presented such an approach using a KDEL peptide linked to an antibody against *c-myc*-tagged

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TGN-38, a transmembrane protein circulating between the *trans*-Golgi network (TGN)<sup>1</sup> and the plasma membrane (Luzio et al., 1990). The KDEL peptide contained an N-glycosylation consensus sequence and was linked to the antibody by a disulfide bridge. Antibodies with the linked KDEL peptide were partially translocated to the TGN, where the peptide could be split from the antibody by treating the cells with DTT. The fact that some of the peptide molecules became N-glycosylated indicates that the peptide had indeed reached the ER. The approach requires the expression of the TGN-38 fusion protein and treating the cells with DTT.

We have applied a different approach by making use of cholera toxin (CTX), which consists of an isopentameric B subunit (CTX-B) (molecular mass of the protomer = 11 kD) and a 28.5-kD A subunit (CTX-A) (Gill, 1976; Mekalanos et al., 1983). The A subunit is synthesized as a single chain but split proteolytically between serine<sub>194</sub> and methionine<sub>195</sub> into the NH<sub>2</sub>-terminal A1 subunit (CTX-A1) (molecular mass = 23 kD) and the COOH-terminal A2 subunit (CTX-A2) (molecular mass = 5.5 kD). Both subunits remain linked together by a disulfide bridge between cysteine<sub>187</sub> of CTX-A1 and cysteine<sub>5</sub> of CTX-A2. CTX binds to the cell surface by interaction of the B subunit with the ganglioside GM1. The bound toxin undergoes endocytosis (Tran et al., 1987; Sofer and Futerman, 1987) and reaches the Golgi (Janicot and Desbuquois, 1987; Orlandi et al., 1993; Nambiar et al., 1993). Although binding of CTX to the plasma membrane and internalization occurs very rapidly, the increase in cAMP resulting from ADP ribosylation of G<sub>sa</sub> by the free A1 subunit of CTX does not start earlier than 20–30 min after application of the toxin (Orlandi et al., 1993; Nambiar et al., 1993), indicating that it is highly unlikely that free CTX-A1 is released from the CTX during the initial binding of the toxin to the plasma membrane.

*Pseudomonas* exotoxin, which enters cells also by an endocytotic pathway, becomes considerably more toxic when applied as a fusion protein with a COOH-terminal KDEL sequence (Seetharam et al., 1991). This effect is significantly reduced in the presence of brefeldin A. Similarly, the toxicity of the ricin A chain, which is also taken up by an endosomal pathway, is strongly enhanced (250-fold in Vero cells) if it contains a COOH-terminal KDEL sequence (Wales et al., 1993). These results could indicate that toxins entering the cells via an endosomal pathway and possessing a COOH-terminal KDEL sequence interact with the KDEL receptor in the Golgi and eventually reach the ER via retrograde transport.

Therefore, we have examined whether CTX or its A subunit can undergo retrograde transport from the Golgi to the ER by means of the COOH-terminal KDEL signal of the A chain. Using CTX with Cy3-labeled B subunits and specific antibodies directed against the CTX-B, the CTX-A, and a peptide of CTX-A2, and applying appropriate incubation and washing techniques, we show here by immunofluorescence studies and subcellular fractionation experiments that CTX-B becomes separated from CTX-A either

before or in the Golgi. The released B subunit is transported to the lysosomes, whereas the released CTX-A chain moves further to the intermediate compartment and to the ER. These results suggest the possibility of using CTX for studying the retrograde transport of the KDEL proteins from the Golgi to the ER and factors regulating this transport. Using this approach, we can show that treatment of Vero cells with nocodazol, given after arrival of CTX in the Golgi, strongly inhibits the transport of CTX-A from the Golgi to the ER and significantly delays the increase of cAMP.

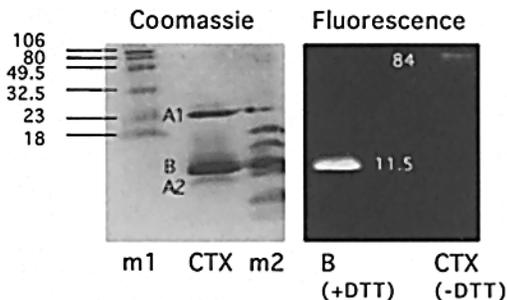
## Materials and Methods

### Materials

CTX and CTX-A subunit were obtained from List Biologicals (Campbell, CA). CTX-B was purchased from Calbiochem (La Jolla, CA) and Sigma (Munich, Germany). The ganglioside GM1 (bovine brain) was from RBI, Natick, MA), nocodazol was from Sigma, and NHS-LC-biotin was from Pierce Chemical Co. (Rockford, IL). UDP-[<sup>3</sup>H]galactose was obtained from Amersham-Buchler (Braunschweig, Germany). CTX and an anti-CTX-A2 antibody were labeled at room temperature for 30 min with a 10-fold molar excess of the sulfoindocyanine dyes Cy3 and Cy5, respectively (Biological Detection Systems, Pittsburgh, PA). Under the labeling conditions used, only the B subunits of CTX became labeled (Fig. 1). All other reagents of analytical grade were from Boehringer Mannheim GmbH (Mannheim, Germany), Merck (Darmstadt, Germany), Biomol (Ilvesheim, Germany), or Serva (Heidelberg, Germany). The peptide NH<sub>2</sub>-KRQIFSGYQSDIDITHNRI-COOH from cholera toxin A2 chain and the peptide NH<sub>2</sub>-CLYITKVLKGGKLSLPA-COOH, representing the COOH terminus of the KDEL receptor ERD2 plus an NH<sub>2</sub>-terminal cysteine, were synthesized by B. Schmidt (Department of Biochemistry, University of Göttingen).

### Antibodies

Polyclonal antibodies against CTX-B were raised in rabbits, polyclonal antibodies against CTX-A, CTX-A1, and against a peptide of the CTX-A2 subunit were raised in guinea pigs and rabbits. The guinea pig antibodies



**Figure 1.** Exclusive labeling of the CTX-B subunits after the reaction of holo-CTX with the succinimide ester of the sulfoindocyanine dye Cy3. (*Left panel*) Coomassie blue staining, *m*<sub>1</sub> and *m*<sub>2</sub>, high and low molecular weight markers, respectively; *CTX*, CTX subunits A1, B, and A2 (reducing gel). (*Right panel*) Cy3-labeled CTX was separated by reducing and nonreducing SDS-PAGE, and the fluorescence of the chromatogram was recorded using a long-pass 590-nm filter (Schott) and an excitation wavelength of 514 nm. Note that fluorescence is associated only with CTX-B (reducing gel) or with the holo-toxin (nonreducing gel), but not with CTX-A. Under nonreducing conditions, the samples containing SDS were not heated. Under these conditions, the holo-toxin (CTX) did not dissociate. Cy3-labeled CTX-B therefore appears (*right panel*) with an apparent molecular mass of ~84 kD.

1. *Abbreviations used in this paper:* CTX, cholera toxin; CTX-A, CTX A subunit; CTX-A1, CTX A1 subunit; CTX-A2, CTX A2 subunit; CTX-B, CTX B subunit; NEM, *N*-ethylmaleimide.

against the CTX-A2 peptide were further purified on protein A-Sepharose (Pharmacia, Freiburg, Germany) and affinity purified on a CTX-A2 peptide column. Antibodies against CTX-B were preadsorbed with CTX-A. Rabbit polyclonal antibodies against the COOH terminus of ERD2, calreticulin, and against the COOH-terminal tetrapeptide KDEL were a kind gift from Dr. Nguyen Van (Abtig. Klin. Biochemie, Univ. of Göttingen), rabbit and goat polyclonal antibodies against cathepsin D were a kind gift from Dr. A. Hille-Rehfeld (Department of Biochemistry, University of Göttingen). The mAbs against the intermediate compartment marker p53 (ERGIC-53) and the Golgi marker giantin were kindly provided by Dr. H.P. Hauri (Biozentrum, University of Basel, Basel, Switzerland). An antibody against  $\alpha$ -tubulin was obtained from Sigma. Secondary antibodies labeled with FITC or tetramethylrhodamine came from Dianova (Hamburg, Germany).

### Immunofluorescence Studies

Vero cells were grown on uncoated coverslips to confluency. The cells were washed with PBS to remove the serum and incubated for 20 min at 0°C with 1  $\mu$ M ganglioside GM1 dissolved in DME without FCS. After prebinding of GM1, the medium was removed and the culture dishes were placed on a thick metal plate that had been cooled to 0°C. The cells were then incubated for 20 min at 0°C with FCS-free DME containing 0.1  $\mu$ g/ml of CTX or Cy3-labeled CTX (only B subunit labeled) to allow binding of the toxin to the plasma membranes. Subsequently, the sample was warmed for 5 min to 37°C to start internalization of CTX. To remove as much free CTX as possible, the cells were rapidly washed three times with PBS containing 1  $\mu$ M ganglioside GM1. After the addition of DME with 10% FCS, the incubation was continued in an incubator (5% CO<sub>2</sub>) at 37°C. At the time points indicated, the coverslips were removed and the cells were fixed in 0.2% glutaraldehyde/2% paraformaldehyde for 10 min on ice and for 20 min at room temperature to prevent toxin translocation during fixation. Free aldehyde groups were blocked with 50 mM NH<sub>4</sub>Cl followed by permeabilization with 0.15% (wt/vol) saponine in blocking medium (PBS/0.2% (wt/vol) gelatin). This was followed by incubation with the first antibody for 30 min at 37°C in the presence of blocking medium. After washing and incubation with the second antibody for 30 min at room temperature, cells were mounted in Moviol 4-88 (Hoechst, Frankfurt, Germany) and dried overnight. Epifluorescence was detected using an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) with a Plan-Neofluar  $\times$ 100/1.30 objective. When Cy3-labeled CTX was used, cells were treated and fixed in the same way, but the time-dependent changes in the distribution of CTX-B were followed by directly measuring Cy3-fluorescence.

In the experiments where nocodazol was used, the cells were first incubated with 0.1  $\mu$ g/ml CTX for 30 min as described above to allow for accumulation of CTX in the Golgi. 10  $\mu$ g/ml nocodazol (final concentration) was then added, and the incubation continued for the indicated times, followed by fixation and immunofluorescence analysis as described above.

### Subcellular Fractionation

Incubation of Vero cells with CTX was performed as described above for the immunofluorescence studies, except that the cells were grown to 90% confluency in 145-cm<sup>2</sup> plastic petri dishes (Greiner, Nürtingen, Germany) in DME/10% FCS. The pretreatment with GM1 and the incubation with CTX were performed as described above for the immunofluorescence studies, except that the concentration of CTX was 0.5  $\mu$ g/ml. At the time points given in the figure legends, the dishes were cooled on ice, the medium was removed, and the cells were washed with ice-cold PBS. The cells were removed with a rubber policeman in the presence of removing medium (140 mM NaCl, 25 mM Tris-HCl, 30 mM KCl, 10 mM EDTA, pH 7.4). After the replacement of the removing medium by homogenization medium (130 mM KCl, 25 mM NaCl, 25 mM Tris, 1 mM EGTA), the cells were homogenized by pushing them three times each through 22G, 24G, and 27G injection needles, respectively. The homogenates were spun for 10 min at 1,000 g. The supernatant was spun for 10 min at 3,000 g, and the resulting supernatant was layered on top of a step gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 10, 7.5, 5 and 2.5% (vol/vol) iodixanol in homogenization buffer. After centrifugation at 126,000  $g_{av}$  for 25 min (SW 40.1 rotor; Beckman Instruments, Palo Alto, CA), 10 fractions were collected from the top of the gradient and analyzed for density, protein (Bradford, 1976), as well as the enzymatic activities of UDP-galactosyl-transferase (Verdon and Berger, 1983), rotenone-insensitive NADH-cytochrome C reductase (Sottocasa et al., 1967), and  $\beta$ -hexosaminidase (Hall et al., 1978). To separate the particulate fractions from soluble proteins, 0.5 ml of each fraction was diluted with PBS and spun for 2 h at 100,000  $g_{av}$ .

The resulting sediments were dissolved in Laemmli mix (Laemmli, 1970) under nonreducing or reducing (50 mM 2-mercaptoethanol plus 50 mM DTT) conditions and analyzed by SDS-PAGE (16% gels) using a multiphasic buffer system according to the method of Wiltfang et al. (1991) to detect free CTX-A2. Only the samples processed under reducing conditions were heated in the Laemmli mix before electrophoresis. In some experiments, the homogenization medium contained 5 mM NEM to alkylate GSH and other sulfhydryl metabolites that could potentially lead to a cleavage of the CTX-A-sulfhydryl bridge.

### Biotinylation of Plasma Membranes

CTX uptake was started as described above for the subcellular fractionation studies. 60 min after the initiation of CTX uptake, the cells were cooled on ice, washed with ice-cold PBS, and incubated for 10 min at 0°C with 0.1 mg/ml NHS-LC-biotin in PBS. Then the medium was removed, and remaining reactive NHS-LC-biotin was blocked by addition of 100 mM Tris-Cl, pH 8. The cells were subsequently homogenized and fractionated by iodixanol density gradient centrifugation as described above. Aliquots of the fractions were diluted and spun for 1 h at 100,000  $g_{av}$ . The sediments were processed by SDS-PAGE and immunoblotting using streptavidin-peroxidase for detection.

### Trypsin Digestion

Cells were allowed to take up CTX as described above for 120 min. They were then homogenized and separated by iodixanol gradient centrifugation as described. The fractions containing the peak concentrations of calreticulin and CTX-A were diluted and sedimented by centrifugation for 2 h at 100,000 g. The sediment was resuspended in 130 mM KCl, 20 mM NaCl, 20 mM sucrose, 25 mM Hepes NaOH, 1 mM Mg acetate, pH 7.4 (medium R) containing 1 mM tetracaine for membrane stabilization. After another centrifugation, the sediments were again resuspended in medium R and incubated for 10 min at 30°C without further addition, or with 30  $\mu$ g/ml trypsin in the absence or presence of 1% (vol/vol) Triton X-100. The samples were suspended in Laemmli mix without reducing agents and were analyzed without previous heating by nonreducing SDS-PAGE.

### Determination of 3', 5'-cAMP

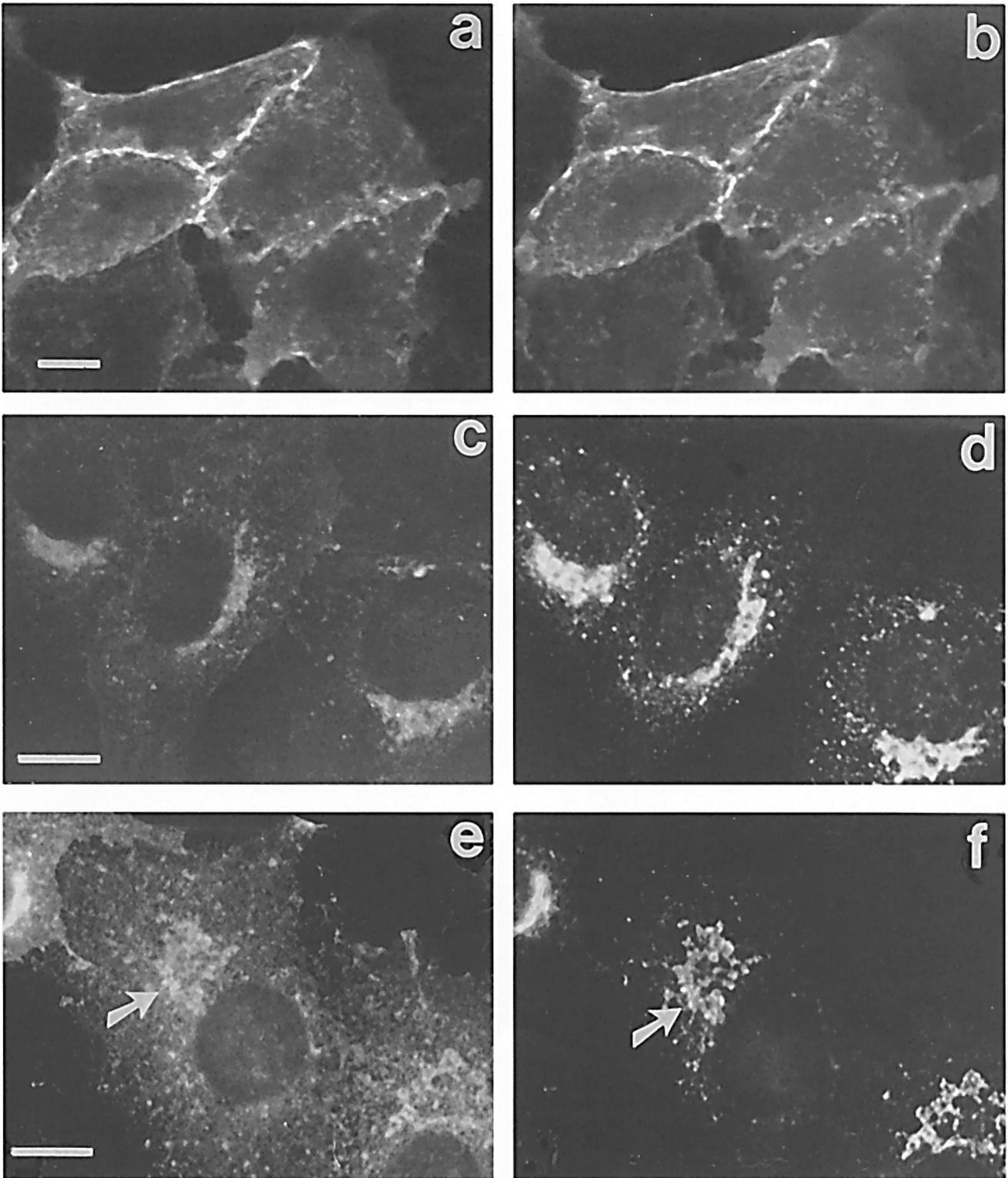
cAMP was measured by an ELISA according to the recommendations of the manufacturer (Amersham-Buchler Co.; code RPN 225).

## Results

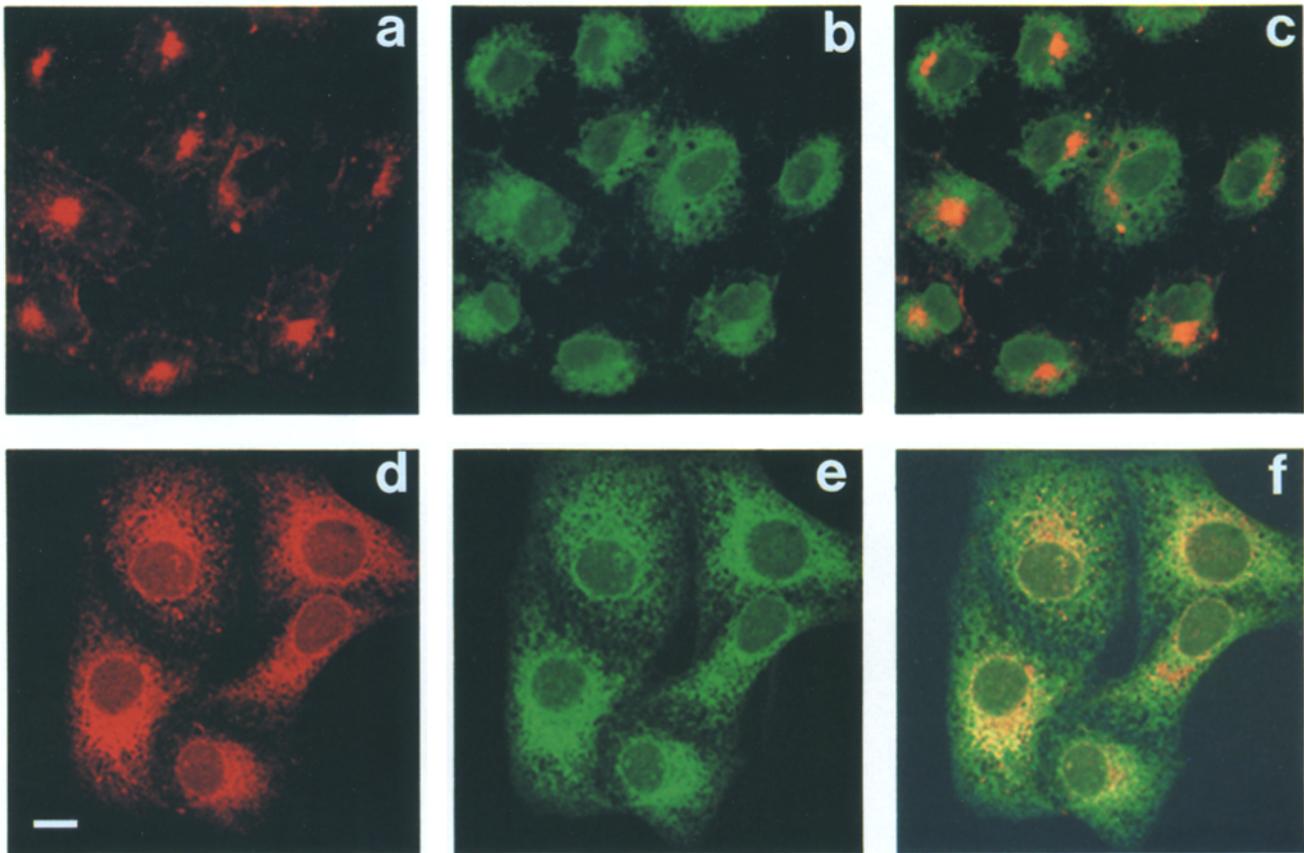
### Immunofluorescence Studies with Complete Cholera Toxin

To exclude the possibility that the uptake of CTX was limited by the number of binding sites in the plasma membranes, the cells had been preincubated with the ganglioside GM1. Since the main goal of the present studies was the transport of CTX within the retrograde secretory pathway, it was not studied whether the preincubation with GM1 accelerated or increased the cAMP response. The washing of the cells with a medium containing GM1 after binding and short internalization of CTX proved to be important for obtaining a clear time-dependent movement of CTX subunits through the different intracellular compartments.

After 5–10 min of incubation with CTX at 37°C, the toxin (A and B subunits) can be observed in association with plasma membranes (Fig. 2, *a* and *b*). At this time, immunopositive small vesicular structures and early staining of Golgi-like structures are also found (Fig. 2 *a*). After 20–30 min, a distinct accumulation of CTX-A (Fig. 2 *c*) in the Golgi occurs, as indicated by the colocalization with the ERD2 protein (Fig. 2, *c* and *d*). At this time, a colocalization of CTX-A with the ER marker calreticulin is hardly detectable (Fig. 3, *a–c*). If the incubation is extended fur-



**Figure 2.** Time-dependent intracellular transport of CTX-A. After 10 min of uptake, significant portions of CTX-A and CTX-B are still associated with the plasma membrane, as detected by the fluorescence of the Cy3-labeled B-subunit (*a*) or by a Cy5 labeled anti-CTX-A-directed antibody (*b*). After 30 min, most of the toxin (as detected with the anti-CTX-A2 peptide antibody) has accumulated in Golgi-like structures (*c*), as indicated by the colocalization with the KDEL receptor ERD-2 (*d*). 45 min after initiation of CTX uptake, CTX-A was no longer restricted to Golgi-like structures (*e*), but it partially colocalized with the ERGIC marker p53 (*f*). Note that *a* and *b* represent confocal laser scan images. Bar, 10  $\mu$ m.



**Figure 3.** Time-dependent translocation of CTX-A from the Golgi to the ER. CTX-A (a), calreticulin (b), and an overlay (c) of a and b 30 min after the initiation of CTX uptake. CTX-A (d), calreticulin (e), and an overlay (f) of d and e 90 min after the initiation of CTX uptake. Laser scan double immunofluorescence. Bar, 10  $\mu\text{m}$ .

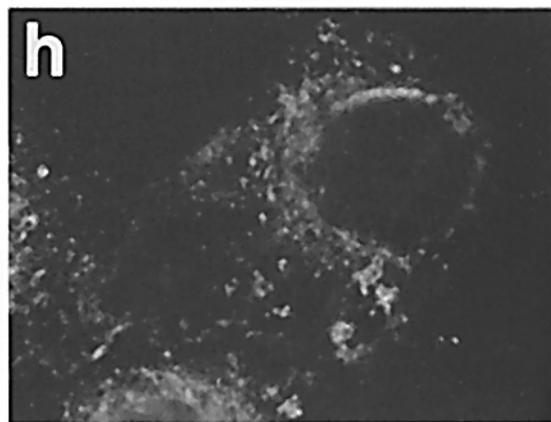
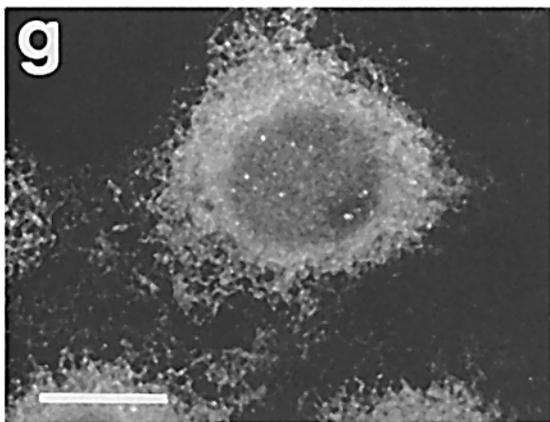
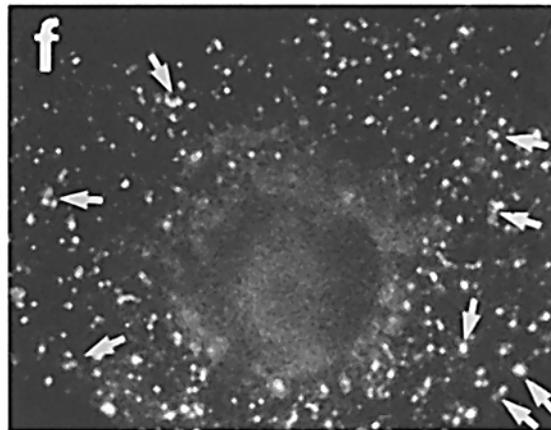
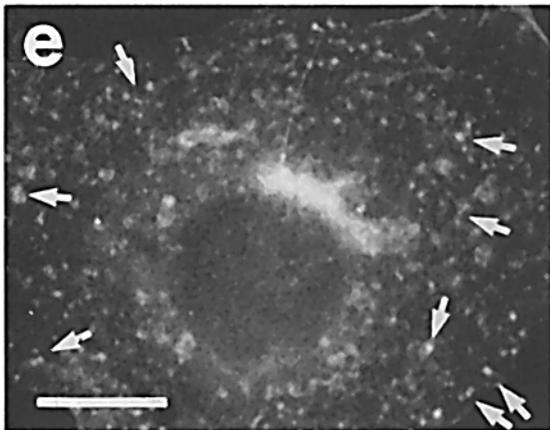
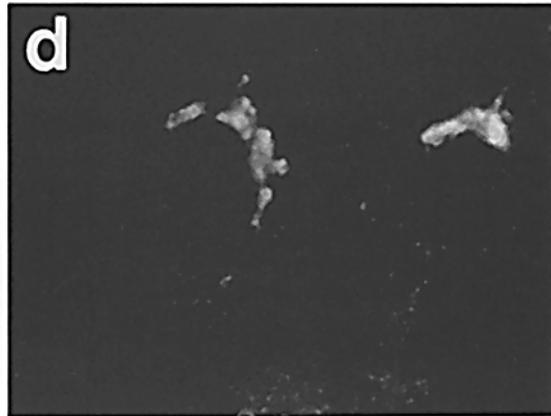
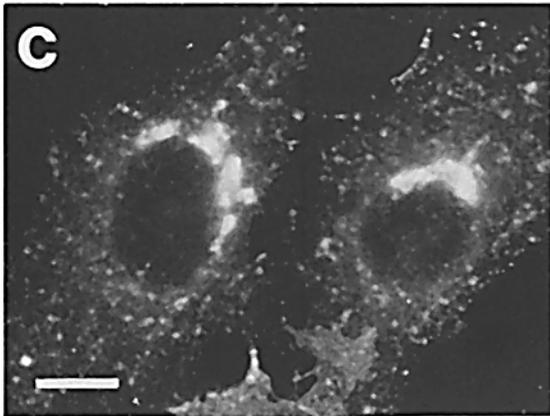
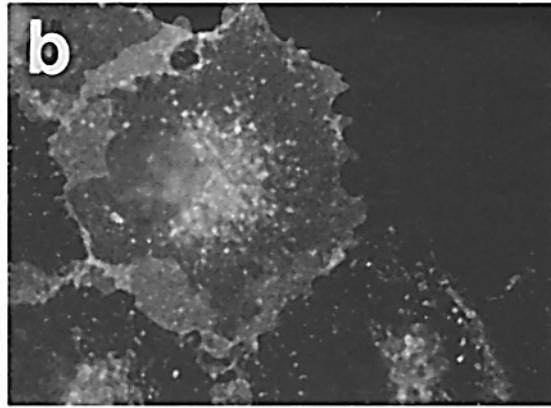
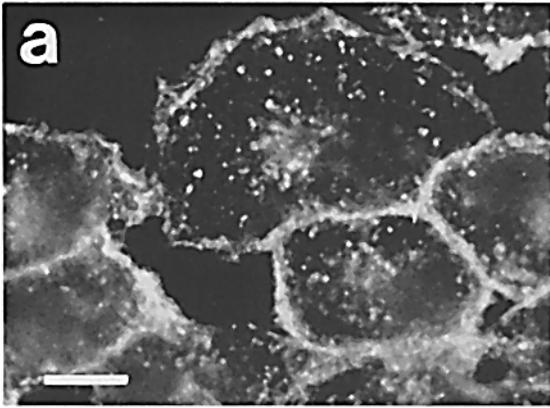
ther, the CTX-A subunit, as assessed with the CTX-A2 peptide-specific antibody, starts to leave the Golgi, as can be deduced from the more extended distribution of CTX-A (Fig. 2 e) and a decreasing colocalization with ERD2 (results not shown here). At this stage, CTX-A partially colocalizes with p53, a marker for the intermediate compartment (Schweizer et al., 1988) (Fig. 2, e and f). After 60–90 min, most of the CTX-A appears in a reticular structure that colocalizes with the ER marker calreticulin (Fig. 3, d–f).

During the early phase of CTX uptake, the transport of the B subunit parallels that of the A subunit. 10 min after initiation of uptake, CTX-B is observed on the plasma membrane and in punctuated structures that most likely represent endosomes (Fig. 4 a). At 20 min, CTX-B starts to accumulate in perinuclear structures (Fig. 4 b). At 30 min, significant amounts of CTX-B colocalize with the Golgi marker giantin (Linstedt and Hauri, 1993) (Fig. 4, c and d), but CTX-B appears also in punctuated structures scattered throughout the cell (Fig. 4 c). At 90 min, when CTX-A exhibits a reticular distribution (Fig. 3, d–f), CTX-B still resides to a large extent in perinuclear compartments (Fig. 4 e), but also in more punctuated structures that colocalize with the lysosomal marker cathepsin D (Fig. 4 f). Clearly, CTX-A does not colocalize with cathepsin D (Fig. 4, g and h). Thus, it appears that CTX-A and CTX-B become separated in the late endosomes or the TGN, from where the

A subunits are further translocated via retrograde transport to the ER while B subunits are channeled into the lysosomes.

#### **Subcellular Fractionation**

The distribution of marker enzymes after iodixanol gradient centrifugation of the 3,000-g supernatant shows a reasonable separation of the Golgi fractions (as represented by UDP-galactosyltransferase) from the ER fractions (as represented by rotenone-insensitive NADH cytochrome C reductase) and calreticulin (Figs. 5 and 6). Fractions 8 and 9 containing most of the ER markers were almost free of contamination with lysosomes. Fractions 8 and 9 together contained only  $\sim 2.5\%$  of total *N*-acetylhexosaminidase activity. Most of the  $\beta$ -hexosaminidase activity was found in the pellet fraction, and some activity was also found in the three top fractions of the gradient. The biotinylation experiments indicate further that the plasma membrane fractions band in the three to four top fractions of the gradient, and that the fractions representing ER, indicated by the distribution of the KDEL protein calreticulin, are free of contaminating plasma membranes (Fig. 6). The last two lanes in Fig. 6 also show that when the 1,000 g supernatant was subsequently centrifuged at 3,000 g, a large amount of biotinylated material could still be sedimented. This material, which to a large extent represents



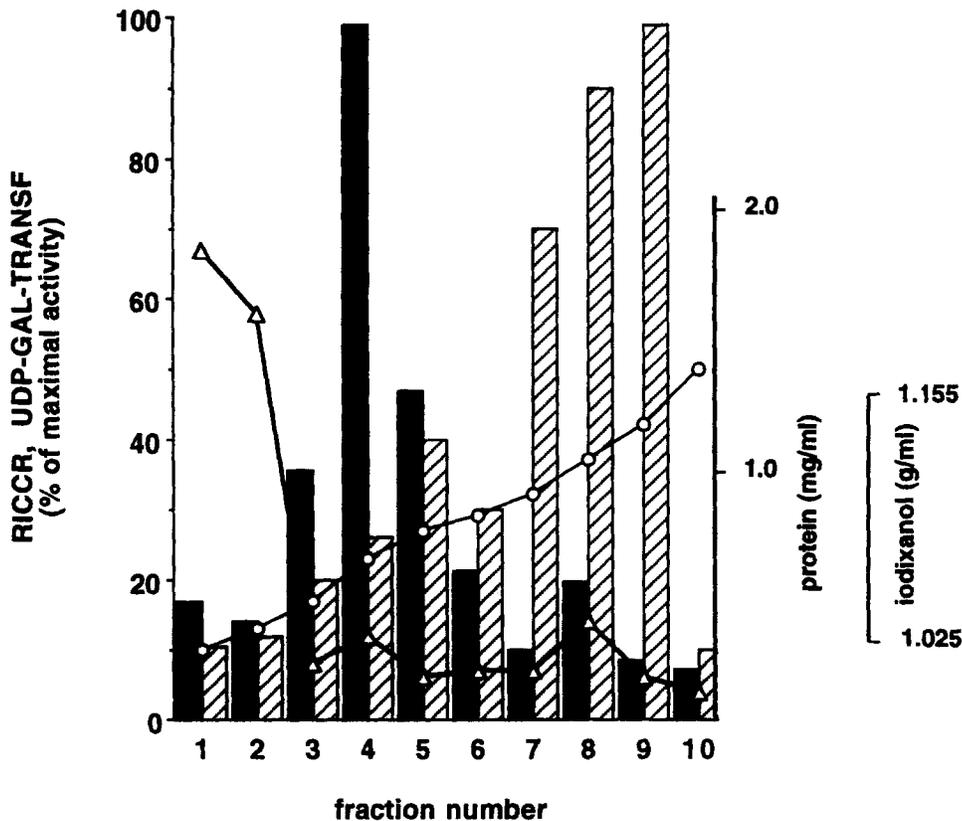


Figure 5. Distribution of marker enzymes. Distribution of the Golgi marker UDP-galactosyltransferase (*UDP-GAL-TRANSF*, black columns) and the ER marker rotenone-insensitive NADH-cytochrome C reductase (*RICCR*, hatched columns) after iodixanol gradient equilibrium density centrifugation of a 3,000-g supernatant of a homogenate from Vero cells. Open triangles, protein concentration; open circles, gradient density. The enzyme activities are expressed as relative activities taking the fraction with the highest total activity as 1.

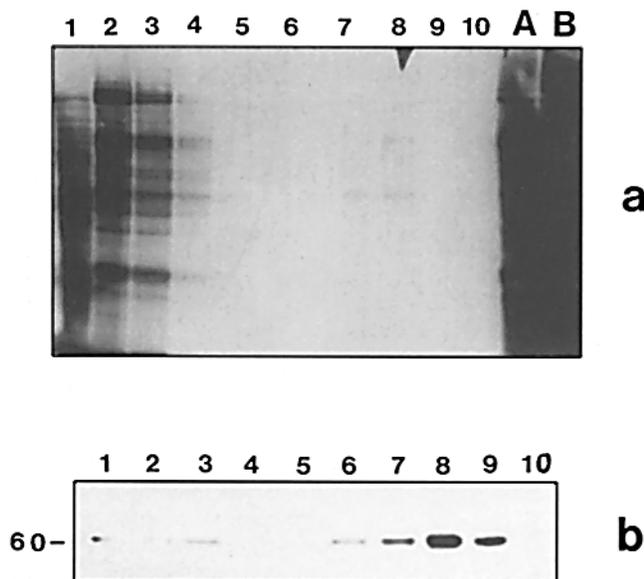
“sheet”-like plasma membranes produced by shear force during cell disruption, had to be removed before gradient centrifugation to yield ER fractions free of plasma membranes.

Cells were analyzed 15 and 60 min after initiation of uptake of CTX for the distribution of CTX-A in the gradient by using the anti-CTX-A2 peptide antibody (Fig. 7). After 15 min, CTX-A appears in a nonreducing gel with an apparent molecular mass of ~28.5 kD and mainly in the first four to five fractions representing plasma membranes, endosomes, and Golgi (Fig. 7a). Clearly, the ER fractions, as represented by the distribution of rotenone-insensitive NADH cytochrome C reductase, are almost free of detectable amounts of CTX-A. At 60 min, however, the nonreducing gel shows CTX-A distributed all over the gradient with maxima in fractions 3–5 and fractions 8 and 9 (Fig. 7b). It runs with an apparent molecular mass of ~28.5 kD, indicating that even the ER contains unsplit CTX-A. Under the same conditions, the CTX-B remains associated mainly with the uppermost four fractions of the gradient and does not appear in the ER fractions (Fig. 7c).

In contrast to the experiments shown in Fig. 7, where an antibody directed against a peptide from CTX-A2 was used, the antibody used in the experiments shown in Figs.

8 and 9 reacted with CTX-A as well as with CTX-A1 and CTX-A2. Fig. 8 indicates that 120 min after the beginning of CTX uptake, free CTX-A1 and CTX-A2 existed in the ER. To exclude the possibility that this reflects an artifact resulting from the leakage of sulfhydryl metabolites (e.g., reduced glutathione, cysteine, cysteamine) into the ER during the homogenization and fractionation procedure, we have performed experiments in which the homogenization medium contained 5 mM NEM (Fig. 9). The experiments were otherwise performed as described in Fig. 7. The fractions were analyzed by nonreducing SDS-PAGE and immunoblotting using the antibody applied also in Fig. 8. After CTX uptake for 30 min, CTX-A, but only traces of CTX-A1, could be detected mainly in the Golgi fractions. Some CTX-A had already reached the ER (as indicated by the position of calreticulin in the gradient, Fig. 9a), but CTX-A1 could not be detected in this compartment (Fig. 9b). After 90 min, a considerable portion of CTX-A had moved to the ER fractions, now accompanied by CTX-A1 as well as by CTX-A2 (Fig. 9c). The minor amounts of sedimentable CTX-A1 and CTX-A2 in the upper fractions most likely resulted from smaller vesicles generated from broken ER cisternae, as indicated by the traces of calreticulin (Fig. 9a) migrating with the same

Figure 4. Experiments with CTX containing Cy3-labeled B subunits: CTX-B does not enter the ER but is directed to the lysosomes. (a–c) Distribution 10, 20, and 30 min after the beginning of CTX uptake, respectively. In d, the cells shown in c were double-stained for the Golgi marker giantin. A comparison of the distribution of CTX-B at 90 min after initiation of CTX uptake (e) with that of cathepsin D (f) indicates that at this time point, a significant portion of CTX-B had reached the lysosomes. At the same time point, CTX-A (as assessed with a CTX-A2 peptide antibody preadsorbed with CTX-B; Fig. 4g) showed little colocalization with cathepsin D (Fig. 4h), but was mainly distributed in reticular structures most likely representing ER (compare with Fig. 3). Bar, 10  $\mu$ m.



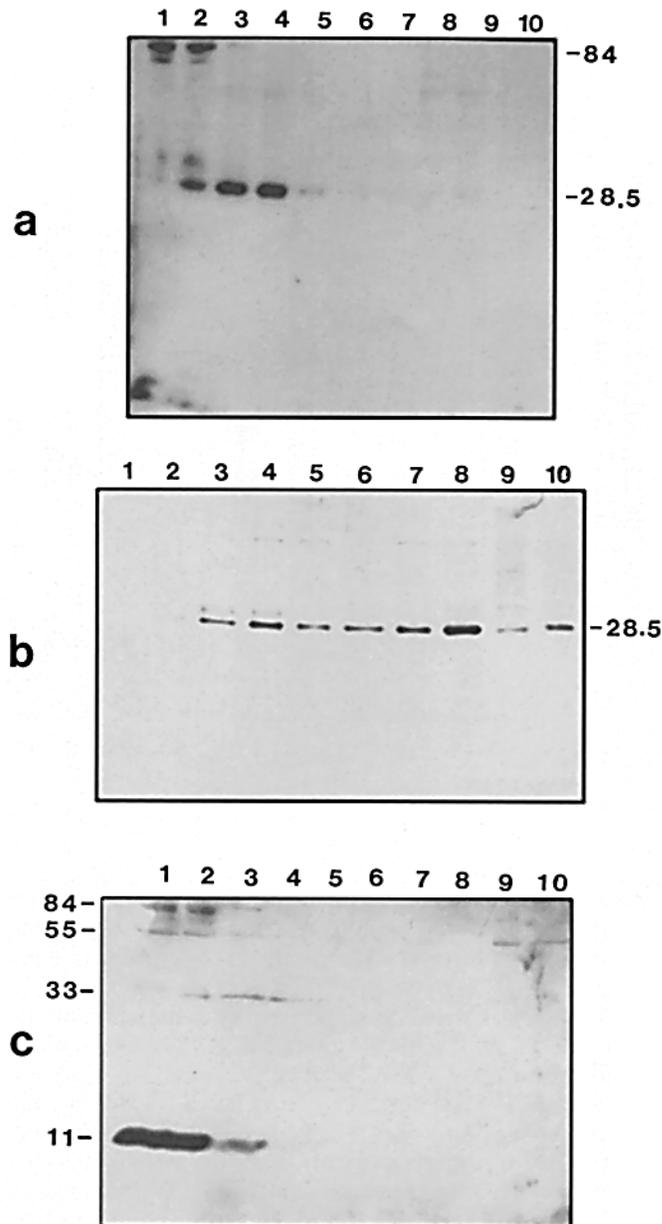
**Figure 6.** Distribution of biotinylated proteins in the iodixanol gradient. Vero cells were labeled with biotin at 0°C for 10 min and homogenized as described in the methods section. The homogenate was spun at 1,000  $g_{av}$  for 10 min, and the supernatant was centrifuged again for 10 min at 3,000  $g_{av}$ . The 3,000-g supernatant was loaded onto an iodixanol gradient and spun for 25 min at 126,000  $g_{av}$  as described in Fig. 6. The fractions were diluted, sedimented by centrifugation at 100,000  $g_{max}$  for 60 min, and the pellets were analyzed by SDS-PAGE. After blotting, biotinylated proteins were detected with streptavidin-peroxidase (*a*). Lanes A and B represent the 3,000-g and 1,000-g sediments, respectively. Note that the 3,000-g sediment still contains significant amounts of biotinylated proteins. (*b*) The distribution of the ER marker calreticulin. Note that some calreticulin moves with the lighter fraction. This represents small ER vesicles generated during cell homogenization.

fractions. These results indicate that after separation from CTX-B, some CTX-A becomes reduced in the secretory pathway, most likely in the ER. Clearly, CTX-A existed inside the ER vesicles since CTX-A, CTX-A1, and CTX-A2 remained resistant to digestion with trypsin as long as the vesicles were not opened by detergents (Fig. 8). After treatment with Triton X-100, the ER marker calreticulin and CTX-A1, but little CTX-A and CTX-A2, became digested by trypsin. Since free CTX-A is very sensitive to trypsin digestion *in vitro* (result not shown here), we conclude that the interactions of CTX-A and the CTX-A2, but not of CTX-A1, with components of the ER protected them from trypsin digestion.

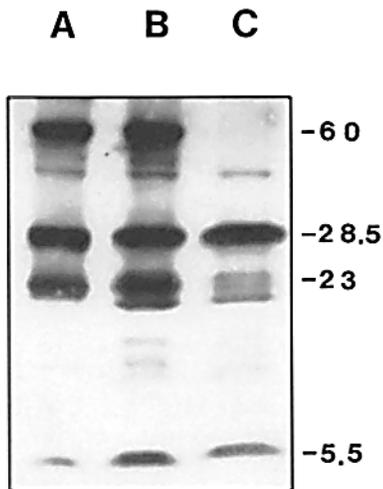
In these experiments, the cells had been homogenized 120 min after initiation of CTX uptake, i.e., 60 min later than in the experiment shown in Fig. 7 *b*. This may explain why A2 subunits could be detected under nonreducing conditions in Fig. 8, but not in Fig. 7 *b*.

#### Time-dependent Increase of 3', 5'-cAMP

The concentrations of 3', 5'-cAMP did not start to increase until 20 min after the beginning of CTX uptake (Fig. 10). A strong increase began only after 30 min, at which time the first CTX-A subunits had already left the Golgi. These results agree with previous data, which also



**Figure 7.** Distribution of CTX-A and CTX-B after iodixanol gradient centrifugation. Distribution of CTX subunits after CTX uptake for 15 min (*a*) or 60 min (*b* and *c*). The initial CTX concentration was 0.5  $\mu\text{g/ml}$ . Vero cells were homogenized 15 min after initiation of CTX uptake. The 3,000-g supernatant was prepared, loaded onto an iodixanol gradient, and centrifuged as described in Fig. 6. The fractions were diluted, spun at 100,000  $g$  for 60 min, and the sediments were analyzed by SDS-PAGE and immunoblotting using the CTX-A2-specific antibody (*a* and *b*) or a CTX-B-specific antibody (*c*). The samples in *a* and *b* were processed by nonreducing SDS-PAGE without heat treatment of the Laemmli mix; the sample in *c* was heated and developed by electrophoresis under reducing conditions. Note that at the early time point (*a*) under the condition of nonreducing electrophoresis of nonheated samples, undissociated CTX could be observed in the first fractions of the gradient, which had disappeared at the later time point (60 min, *b*).

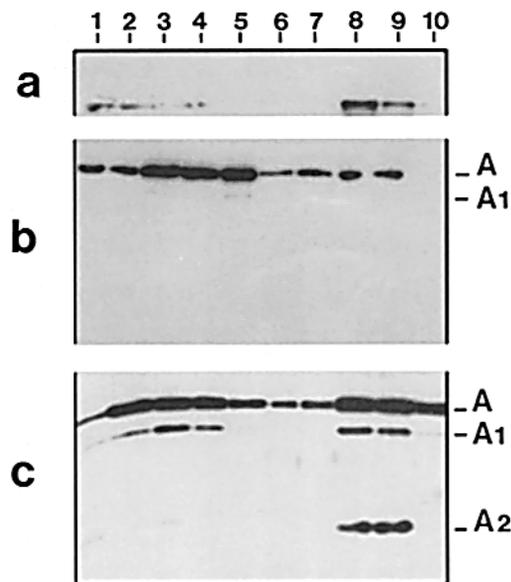


**Figure 8.** Trypsin resistance of CTX-A in the ER. Vero cells were treated with CTX as described in Fig. 7, except that the cells were homogenized 120 min instead of 60 min after the initiation of CTX uptake. The gradient fraction with the highest content of calreticulin was diluted, and the vesicles were sedimented by centrifugation at 100,000 g for 60 min. The sediment was resuspended and divided into three equal portions. The first portion was directly developed by SDS-PAGE under reducing conditions (lane A), the second was first treated with trypsin (lane B), and the third (lane C) was first treated with Triton X-100 followed by trypsin treatment. The positions of calreticulin (60 kD), CTX-A (28.5 kD), CTX-A1 (23 kD), and CTX-A2 (5.5 kD) have been marked. Note that calreticulin and CTX-A1 became digested after treatment with Triton X-100, whereas CTX-A and CTX-A2 remained almost resistant to the action of trypsin, even in the presence of Triton X-100.

showed a delayed response of 3',5'-cAMP levels after CTX application (Orlandi et al., 1993; Nambiar et al., 1993).

#### **Effects of Nocodazol on Retrograde Transport of the CTX-A Subunit**

Nocodazol was applied at a time point (30 min) when most of the CTX had accumulated in Golgi compartments (see Fig. 3). After 20 min of nocodazol treatment (50 min after the initiation of CTX internalization), intact microtubules had disappeared (Fig. 11, a and c), and the compact Golgi structure (as indicated by the distribution of ERD2) had undergone fragmentation to smaller vesicular structures dispersed throughout the cells (Fig. 11, b and d). At this time point, CTX-A was largely found in fragmented structures similar to ERD2 (Fig. 11, f-h). 90 min after the initiation of CTX uptake (60 min after the addition of nocodazol), some CTX-A still colocalized with the fragmented Golgi although the majority of CTX-A appeared in punctuated structures distributed throughout the cell (Figs. 11, i and j). In summary, these results show that nocodazol inhibits the transport of CTX-A out of the Golgi, but is unable to completely block its exit from this compartment. Apparently, the effective translocation of CTX-A from the ERD2-positive (Golgi) compartment to the ER requires intact microtubuli. This would be in line with previous data of Lippincott-Schwartz et al. (1990), who demon-



**Figure 9.** Formation of CTX-A1 and CTX-A2 in the ER. Vero cells were incubated with CTX as described in Fig. 7. 30 min (b) and 90 min (c) after initiation of CTX uptake cells were homogenized as in Fig. 7, except that the homogenizing medium contained 5 mM NEM. The homogenate was spun at 3,000 g, and the supernatant was analyzed by iodixanol gradient centrifugation as described in Figs. 5-7. The fractions from the iodixanol gradient were diluted and spun at 100,000 g for 1 h. The sediments were resuspended in Laemmli mix without heating and were analyzed by nonreducing SDS-PAGE and immunoblotting using an antibody against calreticulin (a) or an antibody against CTX-A that reacted with CTX-A, CTX-A1, and CTX-A2 (b and c). Note the appearance of some calreticulin together with some CTX-A1 and CTX-A2 in the lighter fractions resulting from breakage of ER into smaller vesicles.

strated the involvement of microtubules in the brefeldin A-induced "backtransport" of Golgi enzymes to the ER, as well as the further dependence of this phenomenon on kinesin (Lippincott-Schwartz et al., 1995).

#### **Effects of Nocodazol on the Level of 3', 5'cAMP**

Nocodazol given 30 min after the initiation of CTX uptake led to a significant delay of cAMP increase at 40 min (Fig. 12). After 90 min, however, cAMP levels again achieved similar levels as those measured in cells that were not treated with nocodazol. This agrees well with the immunofluorescence results (Fig. 11), which show that the destruction of microtubules delays the transport of CTX-A out of the Golgi-like structures, but that CTX-A eventually leaves these structures and arrives in more reticular compartments (Fig. 11, i and j).

#### **Discussion**

The approach used here permits the monitoring of the intracellular transport of CTX. This was possible by introducing a short period of CTX internalization followed by more or less complete removal of noninternalized CTX by washing the cells with a GM1-containing medium. Our studies do not permit conclusions as to the internalization

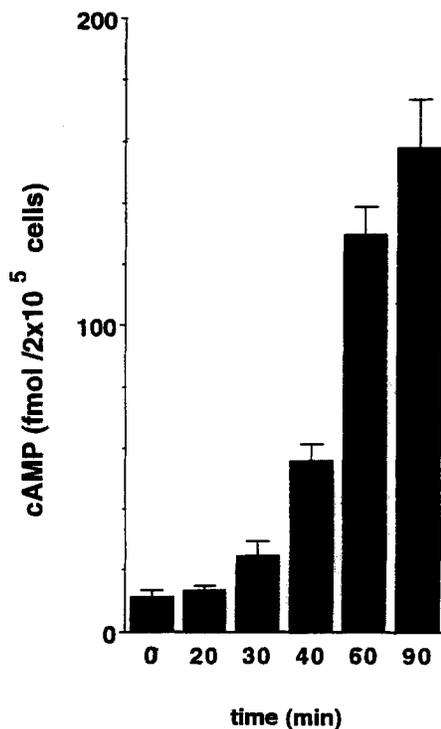


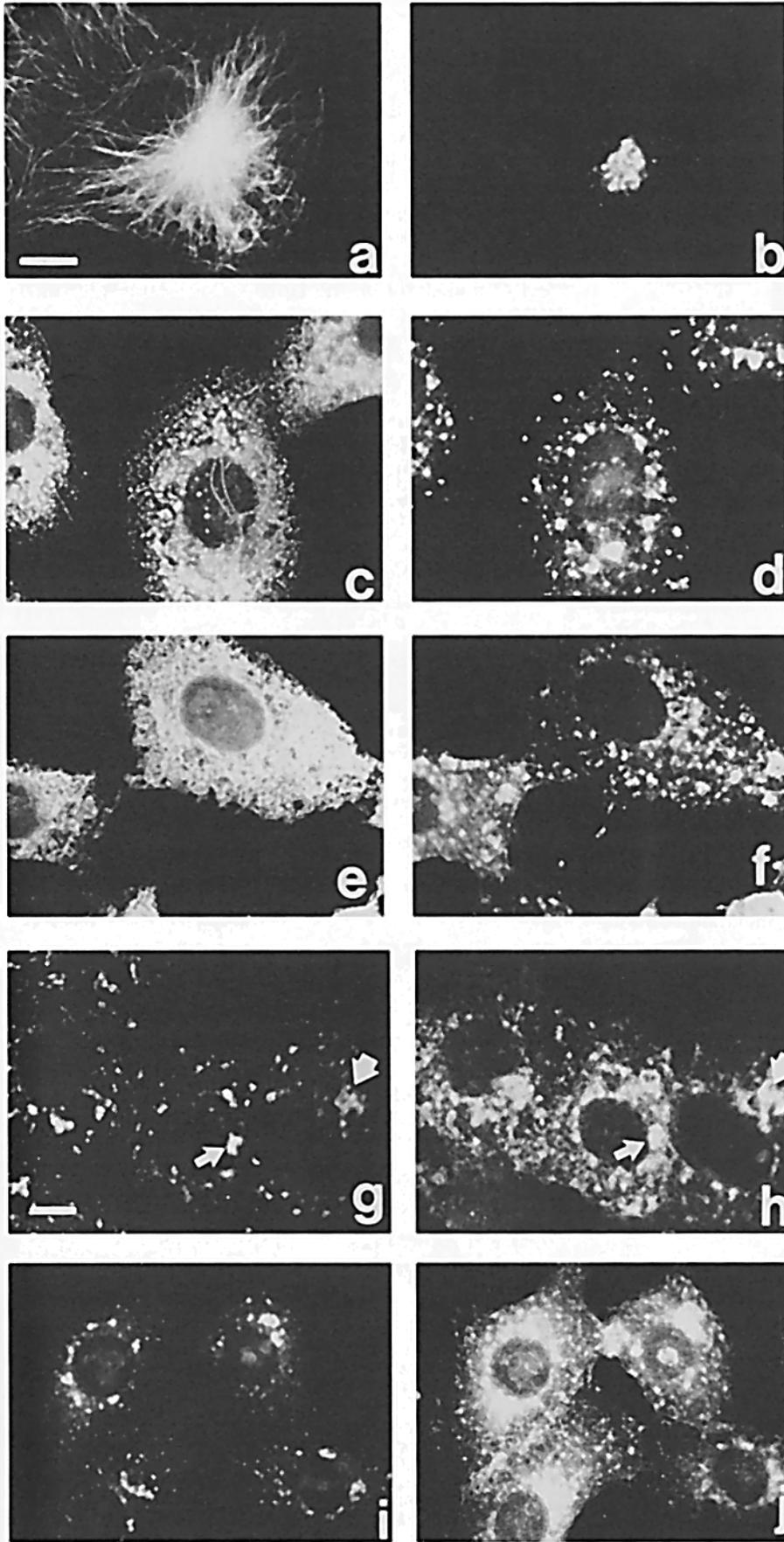
Figure 10. Changes of 3', 5'-cAMP levels in Vero cells after treatment with CTX. Vero cells were treated with 0.1  $\mu\text{g/ml}$  CTX. At the time points indicated, cells were analyzed for 3', 5'-cAMP. Mean values  $\pm$  SD from three separate experiments each point measured in duplicate.

mechanism. It has been reported that after binding to the ganglioside GM1 in the plasma membrane, CTX becomes internalized via noncoated vesicles (Tran et al., 1987), possibly via caveoli (Parton, 1994; Parton et al., 1994), but other mechanisms have been proposed as well (Sofer and Futerman, 1995). In any case, starting  $\sim$ 15 min after internalization, CTX-A and CTX-B appear in the TGN or Golgi compartments, initially as the holotoxin, but later as separated CTX-B and CTX-A, as indicated by measurements of fluorescence resonance energy transfer between the two subunits (Bastiaens et al., 1996). At later time points (60–90 min after the initiation of CTX uptake), a considerable portion of CTX-B colocalizes with the lysosomal marker cathepsin D (Fig. 4), indicating that it had been transported from late endosomes or the TGN to the lysosomes. CTX-B was not observed in the ER at any time point, in accordance with the data of Sandvig et al. (1994), who observed that in A431 cells, CTX-B was taken up by endocytosis and transported to the Golgi, but did not move further to the ER, even under conditions (pretreatment of the cells with butyric acid) leading to the translocation of the *Shiga* toxin B chain to the ER. Since CTX-B was labeled with Cy3 in the immunofluorescence studies (Fig. 4), even proteolytic fragments of CTX-B reaching the intermediate compartment or the ER should have been detected by immunofluorescence. The appearance of CTX in the Golgi is in line with previous reports (Janicot and Desbuquois, 1987; Orlandi et al., 1993; Nambiar et al., 1993), although these studies do not address the question

of a retrograde transport of CTX subunits beyond the Golgi. With respect to the fate of CTX-A, it is important to note that when CTX clearly had already reached the Golgi (10–20 min), the concentration of cAMP remained almost unchanged. The accumulation of CTX-A in the Golgi reached its maximum  $\sim$ 30 min after the initiation of CTX uptake. Thereafter, visible amounts of CTX-A start to leave the Golgi and appear in “downstream” compartments that can be identified as the p53 compartment (intermediate compartment, ERGIC [Schweizer et al., 1988]) and the ER. At the present time, it remains unclear whether CTX-A is first transported to the p53 compartment and then to the ER, or whether most of the CTX-A is transported directly to the ER and a smaller portion to the p53 compartment. In any case, the immunofluorescence studies and the gradient centrifugation experiments showed that CTX-A reached the ER after 60–90 min. The main increase of cAMP levels started after 30 min and did not reach its maximum until 90 min. This observation supports the assumption that an effective ADP ribosylation of  $G_{\text{sec}}$  requires the previous transport of CTX-A from the Golgi to downstream compartments, from where it must be released into the cytoplasm by mechanisms that are still unknown. The small increase in cAMP levels at 30 min does not conflict with this view, since small amounts of CTX-A had reached the ER already at this time point (see Fig. 9 b).

Although we cannot exclude that some CTX-A molecules leave the secretory pathway at the level of the Golgi, it appears from our results, including those obtained with nocodazol, that transport into the cytoplasm from the downstream compartments (intermediate compartment, ER) is more effective. This would also agree with the results that show that *Pseudomonas* exotoxin (Seetharam et al., 1991) and ricin A chain (Sandvig et al., 1994), which are also internalized via an endocytic route, become much more toxic if they contain a COOH-terminal KDEL sequence that presumably facilitates their retrograde transport to the ER. These data seem to be in conflict with recent data of Cieplak et al. (1995). These authors replaced the COOH-terminal RDEL sequence in *Escherichia coli* heat-labile enterotoxin by RDGL, RDEV, or LEDR, and measured the effects of these changes on the cellular elongation of CHO-K1 cells or on cAMP levels in Caco-2 cells. They concluded that the changes of the COOH-terminal tetrapeptide had no significant effects on these parameters. In the experiments with CHO-K1 cells, however, the effect on the morphology was evaluated after the cells had been exposed to the toxins for 36–48 h. This time interval is certainly too long to evaluate the facilitating effect of the RDEL sequence. In the experiments with Caco-2 cells, none of the toxin constructs led to a significant increase of cAMP until 30 min. At 60 and 120 min, the wild-type toxin clearly induced significantly higher cAMP increases than the mutant toxins. Therefore, a functional role of the COOH-terminal KDEL sequence of the A2 subunit of CTX or the RDEL sequence of the A subunit of *E. coli* heat-labile enterotoxin cannot be excluded on the basis of the data presented by Cieplak et al. (1995).

The protease digestion experiments, as well as the cell fractionation experiments in the presence of NEM, show that after 90 and 120 min, not only intact CTX-A, but also



*Figure 11.* Effects of nocodazol on the intracellular transport of CTX-A. Vero cells were treated with CTX as described in Fig. 2. 30 min after the initiation of CTX uptake, nocodazol (10  $\mu\text{g/ml}$  final concentration) was added to the medium, and the incubation was continued for another 50 min. (a and b) Double immunofluorescence of cells in the absence of nocodazol stained for  $\alpha$ -tubulin and ERD2, respectively. (c and d) Double immunofluorescence of  $\alpha$ -tubulin and ERD2, respectively, after nocodazol treatment for 20 min. (e and f) Double immunofluorescence of calreticulin and CTX-A, respectively, after nocodazol treatment for 20 min. (g and h) Double immunofluorescence of ERD2 and CTX-A, respectively, after nocodazol treatment for 20 min. (i and j) Double immunofluorescence of ERD2 and CTX-A, respectively, after nocodazol treatment for 50 min. Bar, 10  $\mu\text{m}$ .

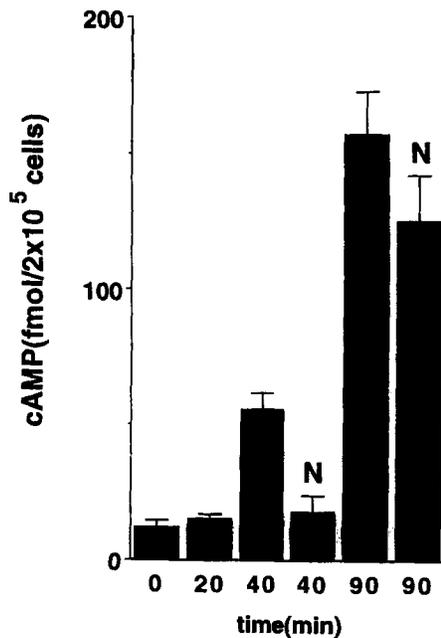


Figure 12. Nocodazol treatment delays the increase of 3', 5'-cAMP levels. The experiments were performed as described in Fig. 11. At the indicated times after initiation of CTX uptake, cells were analyzed for 3', 5'-cAMP. Controls were treated only with CTX. Mean values  $\pm$  SD from three separate experiments each point measured in duplicate.

CTX-A1 and CTX-A2, exist in the ER. The conditions of the NEM experiments were such that all reducing metabolites like reduced glutathione, cysteine, or cysteamine should have become alkylated during the homogenization procedure, so that it seems highly unlikely that CTX-A in the secretory pathway had undergone reduction of its disulfide bridge caused by the influx of reducing metabolites during the homogenization and separation processes. Therefore, the question remains: Where and how does the reduction of CTX-A in the secretory pathway occur? The redox conditions in the ER, where disulfide bridge formation in newly synthesized secretory proteins takes place, is not very favorable for disulfide bridge reduction (reduced glutathione/oxidized glutathione [GSH/GSSG] ratio  $\sim$ 1 [Hwang et al., 1993]) as compared to the GSH/GSSG ratio in the cytoplasm, which amounts to 20–100. Both CTX-A as well as CTX-A1 coexist in the ER. It is not yet clear whether the toxic effect (ADP ribosylation of  $G_{sa}$ ) is exerted by CTX-A1 generated in the secretory pathway or by CTX-A1 produced in the cytoplasm from CTX-A. The fact that CTX-A and CTX-A2 are much more resistant to trypsin digestion in the presence of Triton X-100 than CTX-A1 (Fig. 8) could indicate a preferential interaction of CTX-A and CTX-A2 with the phospholipid bilayer of the ER. Such an interaction could also mean that CTX-A becomes more easily translocated from the ER cisternae to the cytosol than does CTX-A1.

The time-dependent transport of holo-CTX-A allows the study of the backtransport of a KDEL protein from the Golgi to the ER without the dramatic intracellular changes observed during overexpression of the KDEL receptor (Hsu et al., 1992; Townsley et al., 1993), overex-

pression of secretory proteins with a COOH-terminal KDEL sequence (Lewis and Pelham, 1992), or treatment with brefeldin A (Hsu et al., 1992). Our approach is similar to that used by Miesenböck and Rothman (1995) in that it uses an indicator molecule applied to the cells from the outside to study Golgi-ER backtransport, but it is less complicated. Moreover, it allows the monitoring of the transport's time course. This opens the possibility for identifying components involved in discrete transport steps, especially in combination with microinjection techniques. In a first application of the method, we have studied the effect of nocodazol on the Golgi-ER backtransport of the KDEL protein CTX-A. This effect of nocodazol could be discriminated from effects of the drug on earlier transport steps by applying it only after CTX-A, the transport indicator, had reached the Golgi. The results clearly show that disruption of the microtubules inhibits the Golgi-ER backtransport and delays the increase of cAMP. A participation of microtubules in the retrograde transport from the Golgi to the ER would be in agreement with earlier observations of Lippincott-Schwartz et al. (1990) that were obtained in experiments with brefeldin A.

We have now started to study factors involved in the Golgi-ER backtransport of KDEL proteins using the approach described here.

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