

Targeting of Cholera Toxin and *Escherichia coli* Heat Labile Toxin in Polarized Epithelia: Role of COOH-terminal KDEL

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Abstract. *Vibrio cholerae* and *Escherichia coli* heat labile toxins (CT and LT) elicit a secretory response from intestinal epithelia by binding apical receptors (ganglioside G_{M1}) and subsequently activating basolateral effectors (adenylate cyclase). We have recently proposed that signal transduction in polarized cells may require transcytosis of toxin-containing membranes (Lencer, W. I., G. Strohmeier, S. Moe, S. L. Carlson, C. T. Constable, and J. L. Madara. 1995. *Proc. Natl. Acad. Sci. USA*. 92:10094–10098). Targeting of CT into this pathway depends initially on binding of toxin B subunits to G_{M1} at the cell surface. The anatomical compartments in which subsequent steps of CT processing occur are less clearly defined. However, the enzymatically active A subunit of CT contains the ER retention signal KDEL (RDEL in LT). Thus if the KDEL motif were required for normal CT trafficking, movement of CT from the Golgi to ER would be implied. To test this idea, recombinant wild-type (wt) and mutant CT and LT were prepared. The COOH-terminal KDEL sequence in CT was replaced by seven unrelated amino acids: LEDERAS. In LT, a single point mutation re-

placing leucine with valine in RDEL was made. Wt and mutant toxins displayed similar enzymatic activities and binding affinities to G_{M1} immobilized on plastic. Biologic activity of recombinant toxins was assessed as a Cl⁻ secretory response elicited from the polarized human epithelial cell line T84 using standard electrophysiologic techniques. Mutations in K(R)DEL of both CT and LT delayed the time course of toxin-induced Cl⁻ secretion. At T_{1/2}, dose dependencies for K(R)DEL-mutant toxins were increased ≥ 10 -fold. KDEL-mutants displayed differentially greater temperature sensitivity. In direct concordance with a slower rate of signal transduction, KDEL-mutants were trafficked to the basolateral membrane more slowly than wt CT (assessed by selective cell surface biotinylation as transcytosis of B subunit). Mutation in K(R)DEL had no effect on the rate of toxin endocytosis. These data provide evidence that CT and LT interact directly with endogenous KDEL-receptors and imply that both toxins may require retrograde movement through Golgi cisternae and ER for efficient and maximal biologic activity.

RETROGRADE transport through Golgi cisternae has been shown to occur for soluble and membrane proteins of the ER (49) and for certain protein toxins (6, 55). Targeting of soluble ER and some type II membrane proteins in this pathway depends on the COOH-terminal sorting signal Lys-Asp-Glu-Leu (KDEL or HDEL

in yeast) (42, 48, 62). Targeting of type I membrane proteins depends on a short cytoplasmic double lysine motif (19, 20, 43). Retrograde transport of shiga toxin from the cell surface through Golgi stacks to ER appears to depend on the structure of the toxin's cell surface receptor glycosphingolipid Gb3 (56). Movement of shiga toxin along this pathway may be required for toxin action. Likewise, the function of *Pseudomonas* exotoxin A may depend on toxin entry into Golgi or ER as evidenced by sensitivity to mutation in the toxin's COOH-terminal REDLK sequence and rescue by replacement with KDEL (6).

The heat labile enterotoxins produced by *Vibrio cholerae* (cholera toxin, CT¹) and *Escherichia coli* (heat labile

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1. *Abbreviations used in this paper:* CT, Cholera toxin; Isc, short circuit current; LT, heat labile toxin; R, resistance; wt, wild-type.

toxin, LT) also contain respectively the KDEL or the equally potent sorting signal RDEL at the C-terminus of their enzymatically active A subunits (59, 60). CT and LT are structurally, immunologically, and functionally homologous (reviewed in reference 61). Both toxins act directly on intestinal epithelia to elicit massive secretory diarrhea by activating cAMP-dependent signal transduction pathways. Both CT and LT are comprised of five identical B subunits that bind with high affinity to ganglioside G_{M1} at the cell surface (7, 17, 25), and a single enzymatic A subunit comprised of two peptides linked by a disulfide bond (59, 66). After reduction of the A subunit, the enzymatically active A₁-peptide (~22 kD) acts inside the cell to activate adenylate cyclase by catalyzing the ADP-ribosylation of the heterotrimeric GTPase G_sα (5, 39, 40). The A₂-peptide (~5 kD) contains the COOH-terminal K(R)DEL motif that protrudes unhindered below the plane formed by the pentameric B subunit on the side which binds G_{M1} at the cell surface (36, 59, 60).

The presence of K(R)DEL at such a site on both CT and LT raises the distinct possibility that both toxins require direct interactions with endogenous KDEL-receptors for biologic activity (50). The KDEL-receptor is a seven-transmembrane domain protein localized predominantly to the intermediate compartment and Golgi cisternae with a graded distribution from *cis* to *trans* (16, 63). The KDEL-receptor mediates retrograde transport of KDEL-containing proteins from multiple sites within Golgi cisternae including *trans*-Golgi (8, 20, 31, 37, 52).

The process by which CT elicits a response in polarized and nonpolarized cells takes 20–40 min (termed the “lag” phase) (11, 28) and involves movement of CT through multiple intracellular compartments. Binding of CT to the cell surface is followed by endocytosis and subsequent transport of toxin containing membranes to a functionally defined intracellular compartment(s) where reduction and membrane translocation of the A subunit occurs (27, 45). The anatomical compartment of A subunit translocation, however, remains unknown though available evidence suggests this event may occur in Golgi cisternae. For example, CT-labeled horseradish peroxidase or gold particles have been shown to move from the cell surface via endosomes to *trans*-Golgi (23), and recently, a small fraction of internalized CT B subunit has been localized within *cis* elements of Golgi cisternae (56). These data provide direct evidence that CT can move retrograde in the secretory pathway. Neither study, however, provides evidence that transit through Golgi is physiologically relevant to the cellular response.

The lag phase ends with activation of adenylate cyclase by toxin-catalyzed ADP-ribosylation of G_sα. Activation of adenylate cyclase occurs by movement of the reduced and translocated A₁-peptide (or possibly another messenger) back to the plasma membrane. The events that follow reduction (and presumably translocation [24]) of the A subunit are not necessarily diffusion limited. In polarized T84 cells, for example, signal transduction by the reduced A₁-peptide is completely inhibited late in the lag phase by lowering incubation temperatures from 37 to 15°C (28). These and other data (13, 14, 69) suggest that the A₁-peptide may not break free of the membrane after translocation. We have recently found that after translocation the

A₁-peptide may not dissociate from membrane vesicles containing the B subunit. Both subunits may move together via vesicular carriers from the site of translocation to adenylate cyclase on the basolateral membrane (29).

Our aim in the present study was to define intermediary compartments through which CT and LT must physiologically traffic to elicit a cellular response. We examined whether the COOH-terminal K(R)DEL motif of the A₂-peptide may play a role in toxin action. If K(R)DEL were required for normal CT or LT signal transduction, retrograde movement of CT/LT from Golgi to ER would be implied. Recombinant wild-type (wt) and mutant CT and LT were prepared. The COOH-terminal KDEL sequence in CT was replaced by seven unrelated amino acids: LEDERAS. In LT, a single point mutation replacing leucine with valine was made. Toxin action was examined in the human intestinal cell line T84, a polarized cell model of intestinal epithelia and the natural target of CT in nature. T84 cells grown on permeable supports form confluent monolayers of columnar epithelia that exhibit high transepithelial resistance, polarized apical and basolateral membranes, and a cAMP-regulated Cl⁻ secretory pathway analogous to that found in intact intestine (1). Activation of adenylate cyclase in T84 cells by intoxication with CT or LT elicits a strong Cl⁻ secretory response that can be detected using standard electrophysiologic techniques. Our data provide strong evidence that CT and LT interact directly with endogenous KDEL-receptors and imply that both toxins may require retrograde movement through Golgi cisternae and ER for efficient and maximal biologic activity.

Materials and Methods

Materials

Cholera toxin (CT) was obtained from Calbiochem Novabiochem (San Diego, CA). Sulfo-NHS-biotin, sulfo-NHS-acetate, and protein-A-Sepharose were purchased from Pierce (Rockford, IL). All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Hanks balanced salt solution (HBSS, containing in g/L 0.185 CaCl₂, 0.098 MgSO₄, 0.4 KCl, 0.06 KH₂PO₄, 8 NaCl, 0.048 Na₂HPO₄, 1 glucose, to which was added 10 mM Hepes, pH 7.4) was used for all assays unless otherwise stated.

Cell Culture

T84 cells obtained from ATCC were cultured and passaged as previously described (9) in equal parts of DME (1 g/L D-glucose) and Ham's F-12 Nutrient mixture, supplemented with 5% newborn calf serum, 15 mM Hepes, 14 mM NaHCO₃, 40 mg/L penicillin, 8 mg/L ampicillin, and 0.90 mg/L streptomycin. Cells were seeded at confluent density onto 0.33- or 5-cm² Transwell inserts (Costar Corp., Cambridge, MA) coated with a dilute collagen solution as previously described (33). Transepithelial resistances attained stable levels (>1,000 ohms cm²) after 7 d. The development of high transepithelial resistance correlated with the formation of confluent monolayers with well-developed tight junctions as assessed by morphological analysis (32), and with the ability of monolayers to secrete Cl⁻. Cells from passages 71–84 were utilized for these experiments.

Preparation of Recombinant Wild-type and Mutant CT and LT

Mutagenesis of the COOH-terminal K(R)DEL Sequence. The LT-RDEL sequence was mutated in plasmid pTRH29, a derivative of the plasmid vector pBluescript IKS+, that contains the genes for the A and B subunits of *E. coli* labile toxin (70). Mutagenesis of Leu²⁴⁰ to Val at the COOH terminus of the A subunit was performed with an *in vitro* oligonucleotide-

directed mutagenesis kit (Amersham International, Arlington Heights, IL) using single-stranded pTRH29 as a template and a synthetic oligonucleotide (5'-TATTCATACTTCATCC-3', from the Microanalytical Facility, IAPGR, Cambridge Research Station, UK) as the mutagenic primer. The correct Leu to Val substitution was confirmed by dideoxy sequencing using Sequenase II (United States Biochemical Corp., Cleveland, OH) and the resultant plasmid designated pTRH73. The clone producing the CT variant with LEDERAS substituted for KDEL at the COOH terminus of the A subunit was constructed in two steps. First the Lys²³⁷ codon AAG (italicized letters in the sequence GAATTAAG) in pMGJ60 was changed by oligonucleotide-directed mutagenesis to CAG, thereby creating the EcoRI restriction site GAATTC in the resulting plasmid pMGJ67 (22). Next, a PvuII-EcoRI fragment of pMGJ67 that encodes the IPTG-inducible, truncated *ctxA* gene was subcloned into PvuII-EcoRI cut pBR322 to generate pMGJ117, and the predicted fusion joint was confirmed by dideoxy sequencing. Fusion of *ctxA* to vector-derived sequences in pMGJ117 substitutes LEDERAS for KDEL at the COOH terminus of the A subunit of CT, in a manner equivalent to the LEDERAS variant of LT constructed by Sanchez et al. (54). To produce holotoxin with the LEDERAS variant of CT-A, pMGJ117 was transformed into *E. coli* TX1 (Stratagene Inc., La Jolla, CA) containing pMGJ85, a compatible plasmid clone that expresses *ctxB* from the IPTG-inducible promoter of pK184 (21).

Preparation of Periplasmic Extracts Containing Recombinant Wild-type and Mutant CT and LT. *E. coli* XL1-Blue (*recA1 lac- endA1 gyrA96 thi hsdR17 supE44 relA1 [F' proAB lacIq lacZAM15 Tn10]*) (4) or TX1 harboring plasmids encoding wild-type or mutant CT or LT were inoculated from stocks into 200 ml Luria-Bertani medium (L-broth) (53) supplemented with 200 µg/ml ampicillin and 10 µg/ml tetracycline. The cells were grown at 37°C on a rotary shaker. When the culture had reached an optical density of 0.2 (600 nm), isopropyl-1-thio-β-D-galactopyranoside (0.5 mM final) was added and the cells cultured for an additional 2 h. The cells were then collected by centrifugation (6,000 rpm, 4°C for 15 min) and washed in 4 ml of ice-cold PBS (NaCl 140 mM, Na₂HPO₃/NaH₂PO₃ 10 mM, pH 7.2) and then resuspended in 8 ml of ice-cold 0.3 M sucrose, 0.1 M Na₂HPO₃/NaH₂PO₃, pH 7.6. EDTA (5 mM) and lysozyme (10 µg/ml) were added and the cells kept on ice for an additional 15 min with occasional agitation. The treated cells were centrifuged (12,000 rpm at 4°C for 5 min) and the supernatant containing the periplasmic extract removed, dialyzed overnight against two changes of HBSS (1:1,000 vol/vol), separated into 20–50-µl aliquots, flash frozen, and stored at –80°C until required.

Electrophysiology

For electrophysiological studies, confluent monolayers on Transwell inserts were transferred to HBSS and preincubated with apical CT at 4°C for 30 min before shifting to fresh HBSS at 37°C. Measurements of short circuit current (I_{sc}) and resistance (R) were performed with 0.33-cm² monolayers as previously described (9, 28, 33). Serosal and mucosal reservoirs were interfaced with calomel and Ag-AgCl electrodes via 5% agar bridges made with Ringers buffer (114 mM NaCl, 5 mM KCl, 1.65 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 25 mM NaHCO₃, 1.1 mM MgSO₄, 1.25 mM CaCl₂). Measurements of resistance were made using a dual voltage/current clamp device (University of Iowa, Iowa City, Iowa) and 25-µA current pulses. I_{sc} was calculated using Ohm's law.

Selective Cell Surface Biotinylation

Methods described by Sargiacomo et al. (1989) and Le Bivic et al. (1990) were modified for use in the T84 cell system as described (29).

G_{M1} Binding and ELISA

Fluorescein-labeled CT (CT-fluorescein) (28) was used to define binding of recombinant toxins to G_{M1} immobilized on plastic micro-titre plates as previously described (30). Dynatec microfluor 96-well black microtitre plates were coated with type III gangliosides (20 µg/ml; Sigma Chemical Co.) in 60 mM sodium carbonate, pH 9.9, and incubated at 37°C for 36–48 h as previously described (34). After washing in PBS (140 mM NaCl, 10 mM Na₂HPO₃/NaH₂PO₃, pH 7.4) the plates were blocked in PBS containing 5% BSA. CT-fluorescein (3.1 nM in PBS containing 2.5% BSA, pH 7.4) was applied to each well with increasing concentrations (0.05–50 nM) of recombinant wt and mutant toxins or buffer alone, incubated for 30 min at 37°C, washed with PBS, and fluorescence quantified in a fluorescence concentration analyzer (Pandex Fluorescence Plate Reader; Baxter, McGraw Park, IL).

To assay toxin concentrations an ELISA-based assay was used. Periplasmic extracts containing recombinant toxins were applied to 96-well microtitre plates coated with ganglioside G_{M1} as previously described (34). After 30 min at 37°C, the plates were washed with PBS, pH 7.4, and the presence of CT or LT bound to G_{M1} was assayed by routine ELISA using rabbit polyclonal antiserum raised against either CT-A subunit or CT B subunit (27, 29). Toxin concentrations were confirmed by SDS-PAGE and Western blot.

Toxin-catalyzed ADP-Ribosylation of Agmatine

NAD:agmatine ADP-ribosyltransferase activity was assayed as described by Tsai (64) with minor modifications. Recombinant wt or K(R)DEL-mutant toxins (~200 nM final) were incubated at 30°C for 60 min with 15 mM agmatine, 100 µM nicotinamide adenine dinucleotide (NAD, containing 0.02 µCi [*carboxyl*-¹⁴C]NAD), 1 mM GTP, 50 mM KH₂PO₄, 4 mM MgCl₂, 20 mM dithiothreitol, 0.3 mg/ml ovalbumin, 2 mg/ml cardiolipin, 200 µM cibachrome blue, and 20 µl crude guinea pig brain cytosol (as a source of ADP-ribosylating factor) in a final volume of 100 µl. Reactions were terminated with the addition of 1 ml 1% SDS in water and applied to AG 1-X2 (Bio-Rad Labs., Richmond, CA) 0.25 × 2-cm columns. The columns were washed with 4 ml of distilled water and eluates containing hydrolyzed [*carboxyl*-¹⁴C]nicotinamide were assayed by scintillation counting. Recombinant toxins were first activated by incubation with 30 mM dithiothreitol for 60 min at 30°C. Three point calibration was provided by incubation with dithiothreitol activated purified CT (1 or 0.01 µM; Calbiochem Novabiochem) or buffer alone. The assay was linear within these toxin concentrations as assessed by formal dose responses.

Guinea pig brain was homogenized (15 strokes in tight fitting Dounce) in 0.34 M sucrose, 0.1 mM MgCl₂, 1 mM EDTA, 10 mM Hepes, pH 7.4, containing 1.25 mM PMSF and 0.5 µl/ml chymostatin. Cytosol was prepared by differential centrifugation and defined as the supernatant of a 100,000 g spin for 60 min.

SDS-PAGE, Immunoprecipitation, and Western Blotting

Recombinant toxins were analyzed on 10–20% polyacrylamide gradient gels under reducing conditions (unless otherwise indicated for A subunit) and either Coomassie stained or transferred to 0.25-µm nitrocellulose membranes. Rabbit antisera raised against CT-A and CT-B subunits were used as described (29) and diluted 1:200 for immunoprecipitations and 1:1,000 for western blotting. Pre-stained molecular mass markers (Bio-Rad Labs.) contained: lysozyme (18.5 kD), soybean trypsin inhibitor (27.5 kD), carbonic anhydrase (32.5 kD), ovalbumin (49.5 kD), bovine serum albumin (80 kD), and phosphorylase B (106 kD).

Endocytosis

Methods described by Orlandi and Fishman were modified for use in the T84 cell system (44). Recombinant toxins (20 nM) were incubated with confluent monolayers of T84 cells grown on plastic 96-well plates at 4°C for 20 min and then shifted to 37°C or kept at 4°C. At the indicated times, monolayers were returned to 4°C and washed. Toxin remaining at the cell surface was measured by immunoreactivity to anti-serum raised against CT B subunit (1: 200 dilution) (29), secondary incubation with horseradish peroxidase-labeled anti-IgG, and development with 2, 2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid. Assays were performed in triplicate or quadruplicate and reproduced four times. Two point calibrations for each experiment were provided by incubation at 4°C (positive control) and incubations without CT (negative control, ~30% of maximal signal). Means ± SE are reported.

Statistics

Data were analyzed using Statview 512+ software (Brainpower, Inc., Calabasas, CA).

Results

Preparation of Recombinant CT and LT

Mutations were engineered into the COOH-terminal K(R)DEL sequence of the A subunit of *V. cholerae* and *E.*

coli heat-labile enterotoxins. In CT, the COOH-terminal KDEL sequence was replaced by the unrelated seven-amino acid sequence: Leucine-Glutamic acid-Aspartic acid-Glutamic acid-Arginine-Alanine-Serine (Fig. 1). In LT, the COOH-terminal RDEL sequence was altered by a single point mutation replacing leucine with valine (Fig. 1 B). Both removal of KDEL and substitution of valine for leucine have been shown to inhibit post-ER retrieval of K(R)DEL-containing proteins in other eucaryotic systems (47, 48, 71). Recombinant wild-type and mutant CT and LT were expressed from plasmid vectors in *E. coli* XL1-Blue, followed by extraction of periplasmic proteins (containing the recombinant toxins) as described in Materials and Methods. Toxin concentrations were determined by G_{MI} -ELISA against both CT A and pentameric B subunits. Further analysis by SDS-PAGE and Western blot showed that A and B subunits of both mutant and wild-type toxins were present in known concentrations. As assessed by G_{MI} -ELISA, A and pentameric B subunits were present in $\approx 1:1$ ratio indicating that all functional recombinant B subunits were fully assembled with A subunits. Further functional analysis of toxin assembly by competitive inhibition with recombinant B subunit confirmed these results (see below).

Mutation in COOH-terminal KDEL Does Not Affect Toxin Binding or ADP-ribosyl-transferase Activity In Vitro

Toxin binding to G_{MI} immobilized on plastic was examined in vitro to determine whether mutation in K(R)DEL may affect indirectly the ability of the toxin B subunits to associate with cell membranes. Fluorescein-labeled CT was utilized to generate binding isotherms for wt and mutant CT and LT. Fig. 2 shows that both the RDEL- (A) and KDEL-mutant (B) bound to G_{MI} with apparent affini-

ties that were nearly identical to that of wt LT and wt CT ($K_d \approx 6$ nM). Competition with increasing concentrations of heat denatured CT had no effect on CT-fluorescein binding, indicating that binding was specific.

Enzymatic activities of recombinant toxins (not proteolytically nicked in the exposed loop between the A₁ and A₂-peptide) were assessed by in vitro ADP-ribosylation of agmatine (64) (Fig. 3). These data show that wt and KDEL-mutant CT (200 nM) displayed nearly identical ADP-ribosyltransferase activities in vitro (1.6 ± 0.08 vs. 1.8 ± 0.02 nmol NAD hydrolyzed/h, mean \pm SD in triplicate, representative of two independent experiments).

Mutation in the COOH-terminal K(R)DEL Sequence Impedes the Time Course of Toxin Action

Initial experiments with T84 cells showed that mutation in the K(R)DEL sequence delayed the onset of toxin-induced Cl⁻ secretion. At T_{1/2} (half-maximal secretion: 50 min for wt CT and 80 min for wt LT), secretory responses elicited by wild-type toxins were two- to threefold stronger than those elicited by corresponding KDEL- (26 ± 3 vs. $8 \pm 2 \mu A/cm^2$) or RDEL-mutants (25 ± 0.7 vs. $13 \pm 1 \mu A/cm^2$; mean \pm SE, $n = 6-8$). Periplasmic extracts from a control *E. coli* strain XL1-Blue not harboring toxin clones were electrically silent. Mutation in K(R)DEL affected both the initial lag phase and rate of toxin induced Cl⁻ secretion (Fig. 4 and Table I). The inhibitory effect on the kinetics of toxin action appeared greater in monolayers exposed to lower toxin concentrations (Fig. 4, C and D) but was otherwise not dose dependent. At T_{1/2}, dose dependencies for both mutant toxins were increased ≥ 10 -fold (Fig. 5). At high toxin concentrations (100 nM), the maximal secretory responses eventually reached were not significantly different (Fig. 4, A and B).

KDEL-mutants were not degraded by intracellular pro-

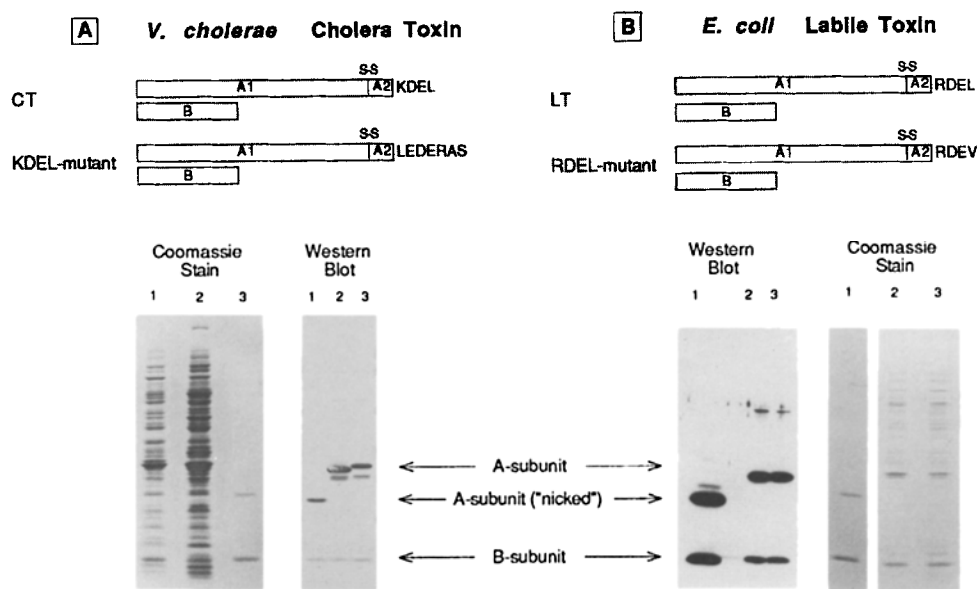


Figure 1. Preparation of recombinant wild-type and K(R)DEL-mutant toxins. Primary structures for wild-type and mutant CT (A) and LT (B) are shown. Recombinant toxins were harvested in periplasmic extracts and analyzed by SDS-PAGE. Coomassie stains A show: periplasmic extracts containing KDEL-mutant CT (lane 1) and wt CT (lane 2); and purified CT from *V. cholerae* (lane 3). Coomassie stains B show: purified CT from *V. cholerae* (lane 1); and periplasmic extracts containing RDEL-mutant LT (lane 2) and wt LT (lane 3). Western blots A show: purified CT from *V. cholerae* (lane 1); and periplasmic extracts containing wt CT (lane 2) or KDEL-mutant CT (lane 3, the KDEL-mutant migrates at a slightly slower rate than wt CT). Anti-sera against A subunit which cross reacts weakly with B subunit was used for western blotting. Western blots B show: purified CT from *V. cholerae* (lane 1); and periplasmic extracts containing wt LT (lane 2) or RDEL-mutant LT (lane 3). Combined anti-sera against A and B subunits were used for western blotting. Both A and B subunits are indicated.

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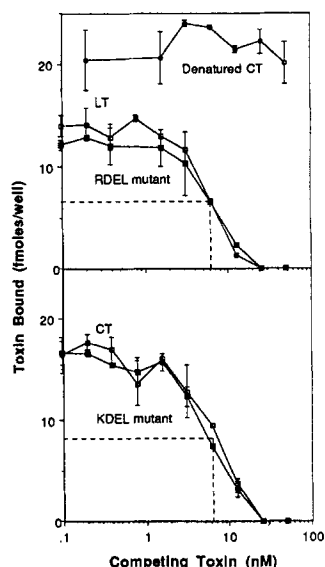


Figure 2. Effect of mutation in K(R)DEL on toxin binding to ganglioside G_{M1} . The fluorescent toxin analogue, CT-fluorescein, was used as tracer as described in Materials and Methods. Binding isotherms for both wt and K(R)DEL mutant toxins were nearly identical and of equally high affinity ($K_d \approx 6$ nM). Representative of two independent experiments in duplicate.

cessing faster than wt CT (Fig. 6). Nearly identical amounts of A and B subunit were immunoprecipitated from T84 cells after 15, 60, and 120 min incubations at 37°C. To exclude the possibility that mutation in K(R)DEL affected toxin action indirectly by affecting assembly of A and B subunits, recombinant LT-B subunits were applied to T84 monolayers in competition with K(R)DEL-mutant or wt toxins (Fig. 7). Recombinant LT B subunit displayed nearly identical IC50's for inhibition of toxin action ($IC_{50} = 10 \pm 4$ nM vs. 10 ± 6 nM, for RDEL-mutant and wt LT, mean \pm SD, $n = 2$ in duplicate, upper panel; and $IC_{50} = 11 \pm 1$ nM vs. 11 ± 4 nM for KDEL-mutant and wt CT, mean \pm SD, $n = 3$ in duplicate, lower panel). These data confirm that mutation in K(R)DEL had no detectable effect on toxin assembly and periplasmic extracts contained little or no free B subunit.

Taken together, these data provide evidence that the K(R)DEL motif is not absolutely required for toxin action, but in its absence signal transduction is significantly less efficient. The clear sensitivity displayed by both CT

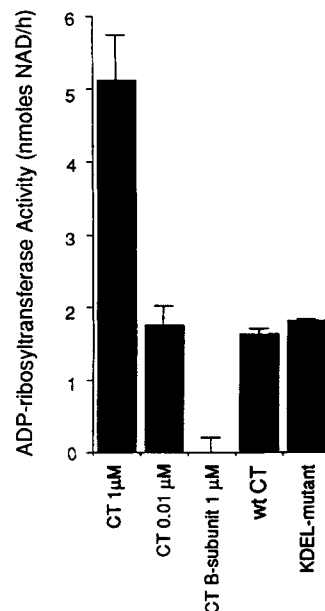


Figure 3. Effect of mutation in KDEL on the enzymatic activity of the A subunit of CT. ADP-ribosyltransferase activity of KDEL-mutant and wt CT (0.2 μ M, not nicked). CT purified from *V. cholerae* (i.e., nicked), at 1 μ M, 0.01 μ M, and vehicle alone provide three point calibration (mean \pm SD, $n = 3$).

and LT to mutation in K(R)DEL strongly implies that both toxins require functional interaction with endogenous KDEL-receptors within Golgi cisternae or ER for efficient retrograde transport and maximum biologic activity.

Effect of Cell Polarity on Signal Transduction by KDEL-Mutants

When applied to basolateral instead of apical reservoirs, CT can bind to the cell at a site in close proximity to its target enzyme adenylate cyclase. Signal transduction from basolateral cell surfaces, however, also requires endocytosis and subsequent transport of CT through multiple compartments of the central vacuolar network (27, 45).

To examine whether signal transduction via basolateral receptors may also require sorting by K(R)DEL, it was necessary to first proteolytically nick (trypsin, 20 μ g/ml) the exposed loop between the A_1 - and A_2 -peptides (residues 187–199) (35). The A subunits of the recombinant toxins are not nicked by endogenous proteases in *E. coli* as they are in *V. cholerae* (Fig. 1, Western blots). This has no effect on the time course of toxin action when applied to apical cell surfaces in the T84 cell system where both recombinant CT and LT are nicked by a protease(s) at the apical cell surface. Nicking, however, appears to be rate limiting when toxins are applied to basolateral membranes (unpublished data).

Signal transduction by toxin internalization via basolateral endosomes was also sensitive to mutation in the K(R)DEL sorting signal (Fig. 8). KDEL-mutants applied to either apical (A) or basolateral cell surfaces (B) elicited a secretory response more slowly than wild-type CT. Both the initial lag phase and rate of toxin induced Cl^- secretion were affected (KDEL-mutant vs. wt CT: lag phase, 53 ± 4 vs. 35 ± 7 min; dI_{sc}/dt , 1.4 ± 1 vs. 2.5 ± 1.4 μ A/cm²/min; peak I_{sc} , 62 ± 25 vs. 80 ± 28 μ A/cm², mean \pm SD, $n = 2$ in duplicate). Identical results (qualitatively) were obtained for wt LT and RDEL-mutants (data not shown). Thus, mutation in K(R)DEL affects a physiologic event in the cellular processing of CT that is in common with toxin entry through apical or basolateral endosomes. This common event displays sensitivity to mutation in the K(R)DEL sorting signal and as such represents retrograde traffic of CT and LT through proximal components of the secretory pathway (compartments shared in polarized cells by the apically and basolaterally directed secretory pathway). Thus these data fit nicely with the current hypothesis that translocation of the A subunit (an event essential for toxin action) may occur within Golgi cisternae or ER (50).

Signal Transduction by KDEL-Mutant CT Displays Greater Sensitivity to Temperature

We examined the temperature dependency of toxin induced Cl^- secretion. We reasoned that if the KDEL sorting signal facilitates a rate-limiting step in toxin action, KDEL-mutants should display greater sensitivity to temperature. Fig. 9 shows the time course of toxin action for KDEL-mutant (1 nM, open squares) and wild-type CT (1 nM, filled squares) at three different temperatures (37, 27, and 20°C). Lowering temperatures below 37°C reduced the rate of toxin induced Cl^- secretion for both wild-type and mutant toxins. Signal transduction by the KDEL-

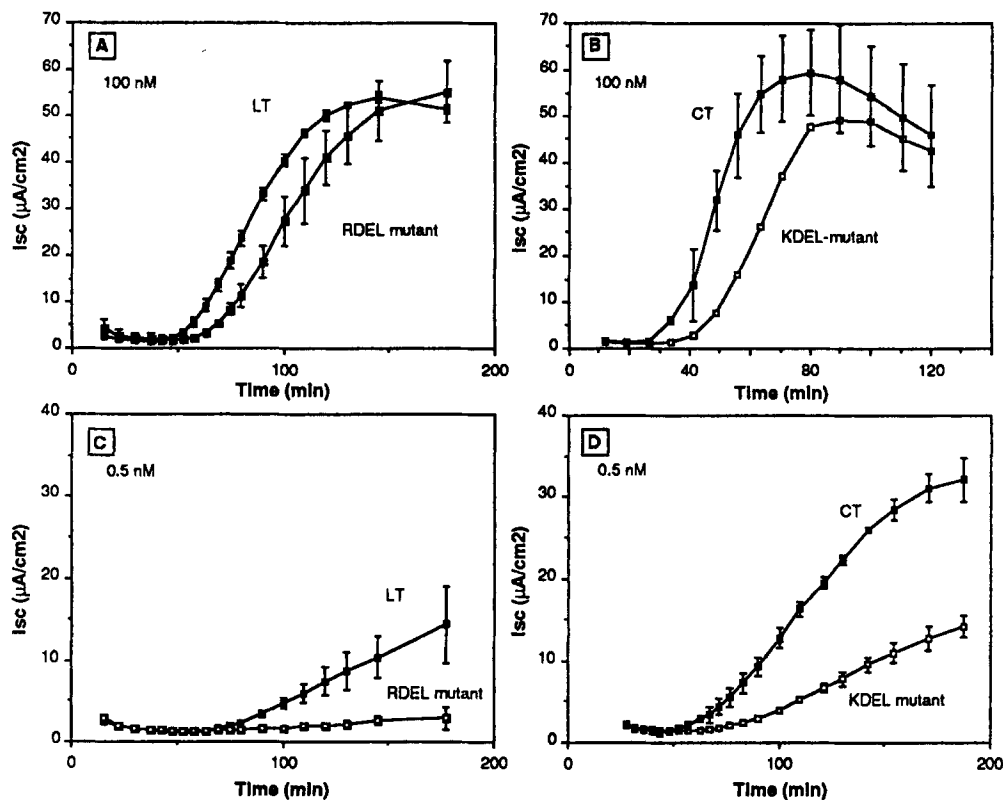


Figure 4. Effect of mutation in K(R)DEL on the time course of toxin induced Cl^- secretion. The time courses of toxin induced Cl^- secretion are shown for LT and the RDEL-mutant in high (100 nM, A) and low doses (0.5 nM, C); and for CT and the KDEL-mutant in high (100 nM, B) and low doses (0.5 nM, D).

mutant, however, was more strongly inhibited by reduced temperature. This is most apparent at 20°C (bottom), where the ability of the KDEL-mutant to elicit a secretory response was completely inhibited, while Cl^- secretion induced by wt CT was clearly present. The viability of these monolayers was demonstrated by the brisk secretory response elicited by the administration of 3 nM vasoactive intestinal peptide to basolateral reservoirs at 480 min (marked with * in lower panel). This differential temperature dependency was not dependent on toxin concentration. Identical results were obtained when wt and mutant toxins were applied in fivefold higher concentrations (5 nM). Table II summarizes the results of seven independent experiments. These data provide additional kinetic evidence that specific interactions between CT and endog-

enous KDEL-receptors occur in human intestine and play a physiologic role in toxin action.

Effect of KDEL-Mutation on Toxin Traffic: Endocytosis Is Unaffected but Transcytosis of the KDEL-Mutant Occurs More Slowly Than for Wild-type Toxin

To show directly that mutation in K(R)DEL affects toxin trafficking, we examined the time course of endocytosis and transcytosis for both wt and KDEL-mutant toxins. The reduced efficiency in signal transduction by K(R)DEL-mutants may be attributable to an effect on toxin uptake by endocytosis. The data show, however, that mutation in K(R)DEL had no detectable effect on endocytosis (Fig. 10). Both wt and K(R)DEL-mutant toxins were internalized at nearly identical rates with apparent half-times of ≈ 7.5 min ($n = 4$, in triplicate or quadruplicate).

The transcytotic movement of wt and KDEL-mutant toxins from apical to basolateral membrane was also examined. In polarized intestinal epithelia, CT must transduce a signal from its apical receptor (G_{M1}) to its basolateral effector (adenylate cyclase). We have recently found that this transcellular signaling may require transcytosis of toxin containing membranes (29). Transcytosis of KDEL-mutant or wt CT from apical to basolateral membranes was assessed directly by selective cell surface biotinylation. These experiments were carried out at 27°C to enhance the difference in time course between KDEL-mutant and wt CT-induced Cl^- secretion.

Fig. 11 A shows the representative results of one of three independent experiments. The upper panel shows a western blot to demonstrate that each lane contains equivalent amounts of immunoprecipitated CT B subunit. The

Table I. Effect of Mutation in K(R)DEL on Toxin Induced Cl^- Secretion

Toxin	Lag phase min	dIsc/dt $\mu\text{A}/\text{cm}^2/\text{min}$	Peak Isc $\mu\text{A}/\text{cm}^2$
CT			
KDEL-mutant $n = 8$	$35 \pm 1^*$	$1.2 \pm 0.06^*$	45 ± 4
Wild type $n = 6$	27 ± 1	1.8 ± 0.1	47 ± 4
LT			
RDEL-mutant $n = 7$	$53 \pm 2^*$	$0.62 \pm 0.03^*$	44 ± 4
Wild type $n = 6$	43 ± 2	0.85 ± 0.03	52 ± 2

Mean \pm SE.

*K(R)DEL-mutant vs. wild-type toxin.

$p = 0.0001$ by ANOVA.

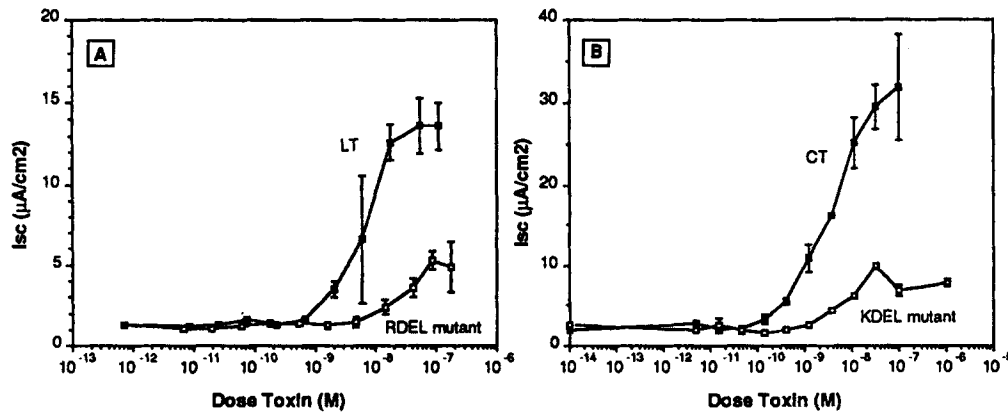


Figure 5. Effect of mutation in K(R)DEL on the dose dependency of toxin induced Cl⁻ secretion. Dose dependency of K(R)DEL-mutant (open squares) and wild-type (filled squares) LT (A) and CT (B). Representative of two to three independent experiments, mean ± SD for duplicate monolayers.

lower panel shows the avidin blot of monolayers exposed to KDEL-mutant or wt CT at 27°C for the indicated times. The first and second lanes are positive and negative controls respectively. Wild-type CT (1 nM) was applied apically to T84 monolayers and incubated for 160 min at 4°C, a temperature that completely inhibits vesicular traffic and CT-induced signal transduction (28). The monolayers were then biotinylated either at the apical (lane 1) or basolateral (lane 2) cell surface. The large avidin-peroxidase signal (lane 1, bottom) demonstrates that CT B subunit can be labeled with biotin while bound to G_{M1} at the cell surface. In the absence of vesicular traffic (at 4°C), however, the CT B subunit was not labeled by biotin to basolateral reservoirs (lane 2). These and supplemental data (29) show that CT does not traverse the T84 cell monolayer by diffusion through intercellular tight junctions.

Lanes 7 and 8 show that in monolayers incubated at 27°C for 160 min basolaterally applied biotin has now labeled a fraction of the B subunit at the basolateral membrane indicating that both the KDEL-mutant and wt CT had breached the epithelial barrier by transcytosis. The fractional component of mutant toxin biotinylated at the basolateral membrane, however, was approximately five-fold less than wt CT. This corresponds quantitatively to the smaller secretory response elicited by the KDEL-mutant at this time point (B). Nearly identical results were obtained for monolayers incubated at 27°C for 110 min (lanes 5 and 6). In contrast, after only 30 min (lanes 3 and 4), neither the B subunit of KDEL-mutant nor wt CT was

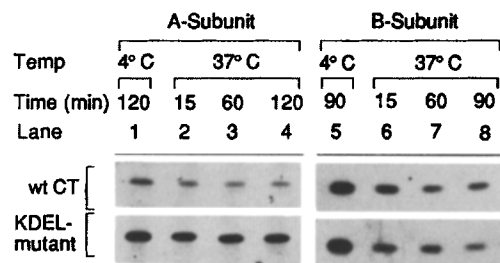


Figure 6. Effect of mutation in K(R)DEL on intracellular degradation. Wild-type (wt) and KDEL-mutant CT were applied to apical membranes of T84 cell monolayers and incubated at either 4°C (lanes 1 and 5) or 37°C for the indicated times. CT A subunits (lanes 1–4) or B subunits (lanes 5–8) were immunoprecipitated from total cell extracts (lysed in the presence of 10 mM N-ethylmaleimide) and analyzed by SDS-PAGE and western blot under non-reducing conditions.

labeled by basolaterally applied biotin at a point in the lag phase where a secretory response was not yet detected. Little or no difference in the amount of B subunit delivered to basolateral membranes was detected in monolayers incubated at 37°C for 90 min (data not shown) or 150 min (A, lanes 9 and 10, and C). Roughly equivalent amounts of mutant and wt toxin were present at the basolateral membrane at these time points which correspond (at 37°C) to nearly maximal secretion for both toxins.

The amount of wt and KDEL-mutant toxin transcytosed were quantified (mean ± SE, n = 3) and displayed superimposed at the appropriate points on the time course of CT-induced Cl⁻ secretion obtained from monolayers of the same passage and studied in parallel (Fig. 11 B). The

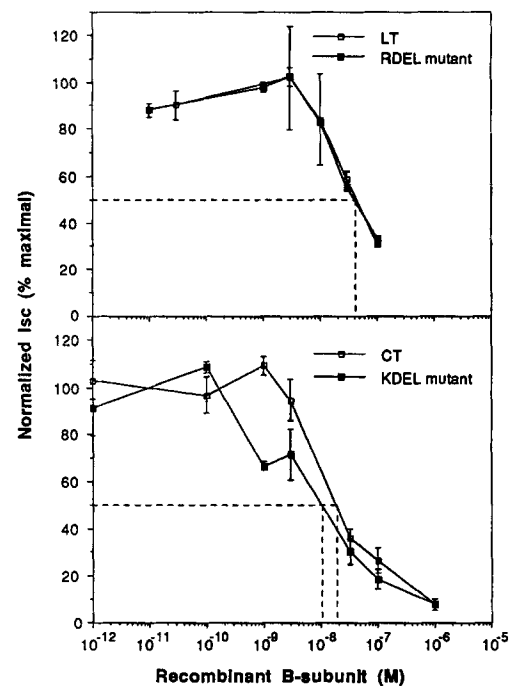


Figure 7. Effect of competition with recombinant B subunit on toxin induced Cl⁻ secretion. Competitive inhibition of secretory response elicited by K(R)DEL mutant (filled squares) and wt (open squares) LT (top) and CT (bottom). I_{sc}'s were normalized to peak secretory responses for each toxin (35 and 45 μA/cm² for RDEL-mutant and LT; 62 ± 25 and 80 ± 28 μA/cm² for KDEL-mutant and CT). Representative of two independent experiments for duplicate monolayers.

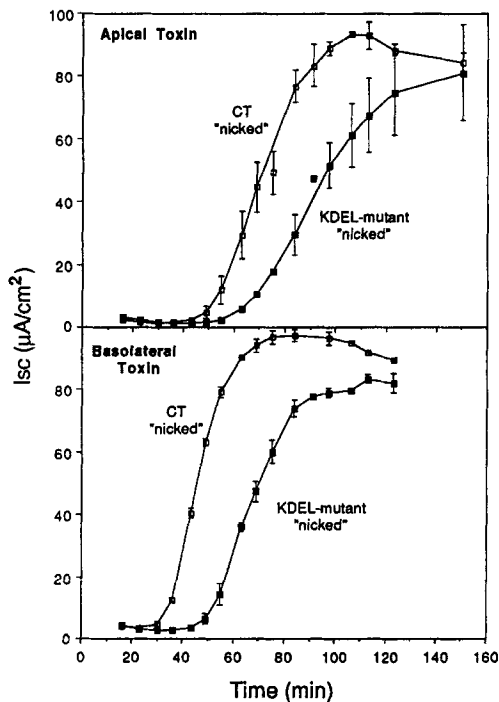


Figure 8. Effect of cell polarity on the time course of Cl^- secretion elicited by KDEL-mutant and wt CT. Time courses of toxin induced secretion are shown for KDEL-mutant and wt CT proteolytically nicked as described in Materials and Methods. Toxins were applied to either apical (*top*) or basolateral (*bottom*) cell surfaces. Signal transduction by KDEL-mutants were delayed from both cell surfaces. Identical results were obtained with RDEL-mutant and wt LT (data not shown). Representative of two independent experiments for both toxins, mean \pm SD for duplicate monolayers.

appearance of the B subunit on the basolateral membrane coincides temporally and quantitatively with mutant (*open squares*) and wt CT-induced Cl^- secretion (*filled squares*). Taken together, these data provide direct evidence that mutation in KDEL affects toxin movement within a basolaterally directed transcytotic pathway and identify Golgi and ER as intermediary compartments through which CT traffics.

Discussion

Evidence for Retrograde Transport of CT and LT into Golgi Cisternae and ER

The results in this paper show that the presence of a functional K(R)DEL sequence in CT and LT plays a role in facilitating toxin action in human T84 cells and thus implies a direct and specific interaction with endogenous KDEL-receptors during signal transduction. In the absence of a functional K(R)DEL sequence at the COOH terminus of the A_2 -peptide, both mutant CT and LT elicited a secretory response more slowly and with higher apparent activation energies than their wild-type counterparts. The interaction between toxin and KDEL-receptor was specific in that a single and conservative point mutation (replacing leucine with valine in the RDEL sequence of LT) affected toxin action. These results were not explained by indirect

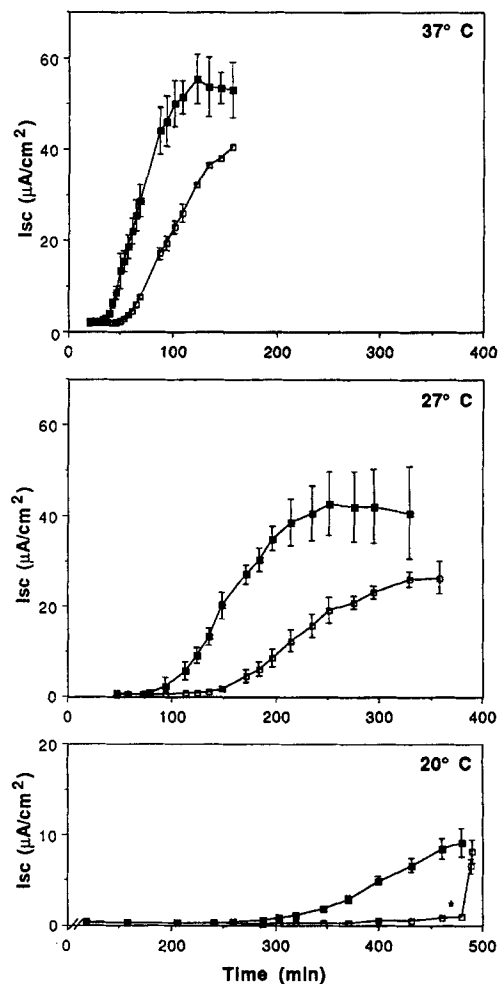


Figure 9. Effect of mutation in KDEL on the temperature dependency of CT-induced Cl^- secretion. Time courses of toxin induced Cl^- secretion are shown for 1 nM KDEL-mutant (*open squares*) and wt CT (*filled squares*) at 37, 27, and 20°C. Representative of 7 independent experiments, mean \pm SD for duplicate monolayers. The * marks the administration of 3 nM vasoactive intestinal peptide to basolateral monolayers at 480 min.

effects on toxin assembly, toxin binding, resistance to intracellular degradation, or ADP-ribosyltransferase activity. In contrast, we provide direct evidence that K(R)DEL effected toxin trafficking as assessed by movement through the basolaterally directed transcytotic pathway. The compartment(s) affected represent a common structure in the apical and basolateral endocytic pathways, as mutation in K(R)DEL inhibited signal transduction from both cell surfaces. These data provide strong evidence that CT and LT may require retrograde movement through the secretory pathway to elicit a maximal secretory response.

Two lines of evidence from the available literature support this view. First, CT (conjugated to horseradish peroxidase) has been visualized directly within *trans*-Golgi in neuroblastoma cells (23) and recently, purified CT B subunit (also conjugated to horseradish peroxidase) has been visualized within *cis*-Golgi in A431 epidermoid cells (56). These studies show directly that CT can traffic from the cell surface to *trans*-Golgi and retrograde into Golgi cisternae. Movement into these compartments appears to de-

Table II. Effect of Temperature on the Secretory Response Elicited by KDEL-mutant and Wild-type CT

	Lag phase	dIsc/dt	Peak Isc	Ratio*Lag	Ratio* dIsc/dt	Ratio peak Isc
	min	mA/cm2/min	mA/cm2			
37°C, n = 7 pairs						
mutant	44 ± 1	0.59 ± 0.04	50 ± 3			
wt	33 ± 1	1.1 ± 0.1	60 ± 5	1.333	0.55	0.82
27°C, n = 7 pairs						
mutant	127 ± 9	0.19 ± 0.02	25 ± 2			
wt	83 ± 2	0.38 ± 0.05	34 ± 3	1.53	0.51	0.74
20°C, n = 7 pairs						
mutant	≥410 ± 20	≤0.012 ± 0.004	2.5 ± 0.8			
wt	256 ± 11	0.48 ± 0.003	10.5 ± 0.6	≥1.6	≤0.25	0.24

Mean ± SE.

*KDEL-mutants are significantly more sensitive to temperature than wt CT.

p = 0.001 by 2-factor ANOVA.

pend on toxin binding (or clustering) ganglioside G_{M1}, as purified or recombinant CT B subunit will move into these compartments in the absence of sorting signals from the A subunit. These data fit nicely with our current findings that the K(R)DEL sequence is not absolutely required for toxin action, and with our concurrent observations (29) that trafficking of CT through a basolaterally directed transcytotic pathway also depends primarily on toxin-binding (or perhaps clustering) ganglioside G_{M1}.

Second, the KDEL-receptor, the product of the mammalian ERD2 gene, has now been clearly localized at steady-state to Golgi cisternae and intermediate compartment with small amounts of receptor visualized in ER and *trans*-Golgi (16, 63). KDEL-receptors were not visualized at the cell surface or in more distal compartments of the

secretory pathway. Furthermore, the preponderance of evidence establishes that KDEL-receptors mediate retrograde transport of other KDEL-containing proteins through Golgi cisternae to ER (42, 48, 62). Retrieval and recycling to ER occurs from multiple sites in the secretory pathway, including *trans*-Golgi (8, 20, 31, 37, 52). Thus, CT and LT would have multiple opportunities to interact with KDEL receptors as these toxins move retrograde in the secretory pathway via binding (or clustering) ganglioside G_{M1} (see below).

Components of the apical and basolateral endocytic pathways converge in late endosomes (2, 12, 18, 46) and almost certainly in the *trans*-Golgi, as vesicles exchange constitutively between these compartments (15 and references therein). Our demonstration, however, that the K(R)DEL sorting signal plays a physiologic role in toxin action indicates that both CT and LT move beyond the late endosome into compartments where interactions between toxin and KDEL-receptors may plausibly occur. Thus, these studies identify *trans*-Golgi, Golgi cisternae, and possibly ER as intermediary compartments through which CT may physiologically traffic to elicit an efficient secretory response. Since reduction and translocation of the A subunit also represent events that occur in shared (or similar) compartments of both the apical and basolateral endocytic pathways (27), it is tempting to speculate that A subunit translocation occurs in ER or possibly Golgi cisternae as previously hypothesized (50). If so, movement into these compartments would be facilitated by direct interaction between toxin and K(R)DEL-receptors. This prediction fits nicely with our experimental data and provides a cogent and biologically plausible explanation for conservation of the COOH-terminal K(R)DEL sorting signal in these two toxins. However, it remains possible that mutation in K(R)DEL may also affect membrane translocation of the A subunit by a mechanism separate from its effect on toxin trafficking.

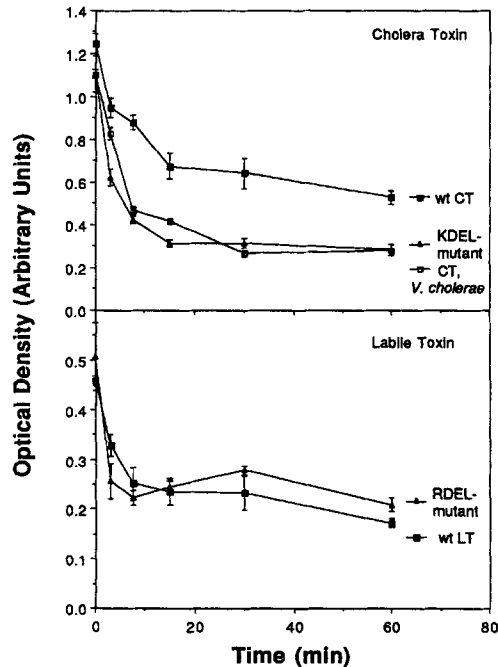


Figure 10. Effect of mutation in K(R)DEL on toxin endocytosis. Time course of toxin endocytosis. (Top) wt CT (filled squares), KDEL-mutant (filled triangles), and wt CT purified from *V. cholerae* (open squares). (Bottom) wt LT (filled squares), RDEL-mutant (filled triangles). Both wild-type and K(R)DEL-mutant toxins display nearly identical rates of internalization with apparent half-times of ≈7.5 min.

Biology of Glycosphingolipids and Targeting of CT/LT by Binding or Clustering Ganglioside G_{M1}

Our current studies, together with those discussed above (23, 38, 56), show that both retrograde traffic into the secretory pathway and transcytosis across polarized cells (29) may plausibly depend on structural features of the toxin's endogenous receptor, ganglioside G_{M1}. Glycosphin-

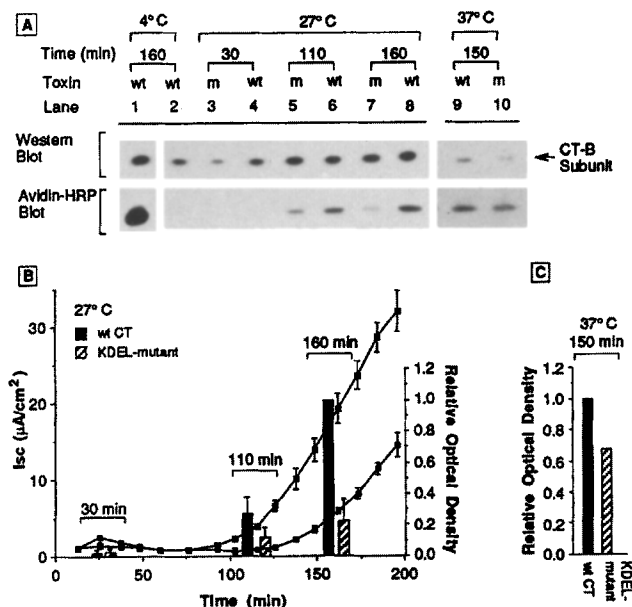


Figure 11. Effect of mutation in KDEL on toxin trafficking through the transcytotic pathway. T84 cell monolayers grown on permeable supports were exposed continuously to 1 nM KDEL-mutant (*m*) or wild-type (*wt*) CT at the apical cell surface at the indicated temperatures and times. Selective cell surface biotinylation was performed as described. (A) *Top* shows western blot of immunoprecipitated CT B subunit showing that each lane contains equivalent amounts of CT B subunit. *Bottom* shows HRP-avidin blot of same experiment. Lane 1, (positive control) 120 min incubation at 4°C, biotinylation from apical cell surface; lane 2, (negative control) 120 min incubation at 4°C, biotinylation from basolateral cell surface; lanes 3–10 were biotinylated basolaterally; lanes 3 and 4, 30 min incubation at 27°C; lanes 5 and 6, 110 min incubation at 27°C; lanes 7 and 8, 160 min incubation at 27°C; lanes 9 and 10, 160 min incubation at 37°C. (B) Time course of KDEL-mutant (*open squares*) and *wt* (*filled squares*) CT-induced Cl^- secretion at 27°C. Relative band optical densities for avidin blots of monolayers incubated at 27°C (mean \pm SEM, $n = 3$) are superimposed at the appropriate time points. Signals in lane 8 (160 min, *wt* CT) and lane 2 (negative control) for each independent experiment served as two point calibrations. (C) Relative band optical densities for avidin blots of monolayers incubated at 37°C for 150 min. Signals in lanes 9 and 2 served as two point calibrations.

golipids such as ganglioside G_{M1} are expressed exclusively on the outer (exo-cytoplasmic) leaflet of cell membranes (reviewed in 57, 65, 67, 68). They contribute to the structure and function of membrane domains throughout the central vacuolar network of eucaryotic cells except perhaps ER where they may be present in extremely low concentrations. Transport between distinct intracellular structures occurs only by vesicular traffic. Thus, ganglioside G_{M1} , which has been shown directly to recycle between the cell surface and *trans*-Golgi in murine NCTC 2071 and rat glioma C6 cells (10), represents an ideal target for toxins entering the endocytic and retrograde secretory pathway. Targeting of CT and LT into Golgi and ER (though not efficient) may simply depend on binding (or clustering) G_{M1} via the pentameric B subunit. This would explain our observations that the K(R)DEL sorting signal is not absolutely essential for toxin action.

Possible Mechanism of CT Action on Polarized Epithelia

Based on our current understanding, we propose the following working model of toxin action on polarized epithelia (Fig. 12). Binding of CT or LT to ganglioside G_{M1} at the apical cell surface is sufficient for targeting these toxins to all intracellular compartments necessary for signal transduction. In the absence of any other targeting information, however, retrograde movement of CT or LT through the endocytic system to more proximal compartments of the secretory pathway is not efficient. Interaction with endogenous KDEL-receptors shifts the steady state distribution of the CT- G_{M1} complex towards Golgi cisternae and ER where reduction and translocation of the A subunit may occur. If after translocation the A_1 -peptide breaks free of the membrane, activation of adenylate cyclase on the basolateral membrane may then occur by diffusion through the cytosol. Available data indicate, however, that the A_1 -peptide may not dissociate from the membrane after translocation (28, 69), and that signal transduction may depend instead on movement of the CT- G_{M1} complex back out the secretory pathway (via anterograde transport) within basolaterally targeted vesicles (29). Escape of toxin from the KDEL-recycling pathway into anterograde transport vesicles may occur as a result of saturation of KDEL-receptors (by incoming toxin) or by sequestration of the K(R)DEL motif after translocation of the A subunit. Fusion of toxin-containing vesicles with the basolateral membrane delivers the A_1 -peptide to a site where the toxin can now catalyze the ADP-ribosylation of G_{α} . Thus, direct interaction with endogenous KDEL-receptors facilitates toxin entry into Golgi cisternae and ER where reduction and translocation of the A subunit may occur and where the toxin may now transfer from apically recycling to basolaterally targeted vesicles (Fig. 12). Both maximal toxin action and efficient transcytosis then depends on trafficking through these proximal components of the secretory pathway.

Though transcytosis of toxin-containing membranes from apical to basolateral cell surface appears to be required for biologic activity, transcytosis of toxin subunits by itself will not lead to signal transduction (29). Our current and previous studies show that CT must traffic through multiple intracellular compartments to elicit a Cl^- secretory response. Here we provide evidence that identifies *trans*-Golgi, Golgi cisternae, and possibly ER as intermediary compartments through which CT may traffic. Available data indicate that this indirect route across T84 cells may be more complex than that taken by the apically directed polymeric immunoglobulin receptor (41) or by basolaterally targeted VSG proteins artificially inserted into apical membranes of MDCK cells (51). As such, we introduce the term “indirect transcytosis” to describe this process.

We can now tentatively assign anatomic identities to physiologic compartments previously defined only by functional criteria. For example, brefeldin A inhibits completely CT-induced signal transduction from both the apical and basolateral cell surface. Brefeldin A scrambles protein targeting and diverts the movement of CT away from a compartment necessary for reduction and translocation of the A subunit (27). The brefeldin block occurs early in the lag

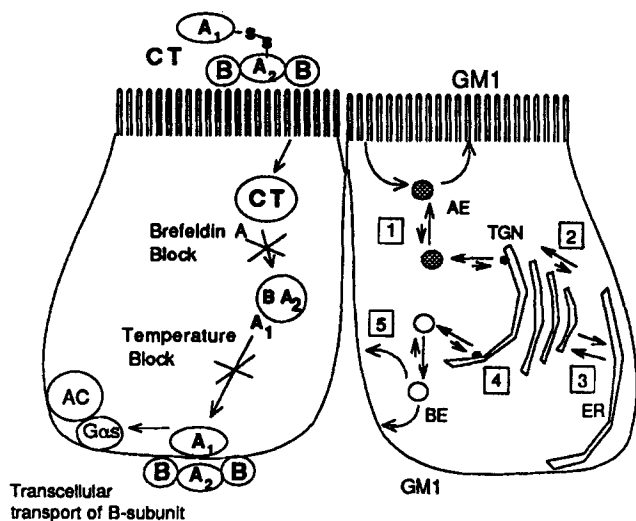


Figure 12. Proposed model of toxin action on polarized epithelia. (Left) Depicts transport steps functionally defined in earlier studies. (Right, 1) Toxin enters the cell by binding G_{M1} at the apical cell surface. The bulk of the CT- G_{M1} complex recycles to the apical cell surface through early and late apical endosomes (shaded structures). (2) The COOH-terminal K(R)DEL sorting signal on the A subunit facilitates retrograde movement of the CT- G_{M1} complex through Golgi cisternae to ER. (3) Reduction and translocation of the A subunit may occur in Golgi cisternae or ER and the CT- G_{M1} complex is sorted (or transferred) from apically recycling to basolaterally targeted membrane structures. (4) Transport to the basolateral membrane occurs by anterograde vesicular traffic back out the secretory pathway. (5) Fusion of basolaterally targeted vesicles carrying the CT- G_{M1} complex delivers the translocated A subunit to a site near the Gs/adenylate cyclase complex on the basolateral membrane. Arrows are scaled to depict the steady state distribution of glycosphingolipids within the central vacuolar network. AE, apical endosome; TGN, trans-Golgi network and Golgi cisternae; ER, endoplasmic reticulum; BE, basolateral endosome; AC, adenylate cyclase; Gas, heterotrimeric GTPase Gs.

phase. Based on our current studies, we propose that brefeldin A may inhibit toxin action by inhibiting retrograde transport into *trans*-Golgi or Golgi cisternae. Either of these sites would be consistent with known effects of brefeldin A on membrane traffic in both polarized and nonpolarized cells (26).

Signal transduction from the apical membrane of polarized cells also depends on movement of CT through a separate temperature-sensitive step (27, 28). This occurs late in the lag phase after the A subunit has been reduced and presumably translocated. This temperature-sensitive step is not apparent when CT is applied to basolateral cell surfaces, and represents a step(s) uniquely required for signal transduction from apical membranes. We propose that this late lag phase temperature-sensitive step represents sorting (or transfer) of toxin from apically derived to basolaterally targeted membrane structures (i.e., membrane microdomains). This must occur within Golgi cisternae or ER as mutation in K(R)DEL has a clear effect on transcytosis of CT. In contrast, CT internalized from basolateral membranes of the same cells does not display such a dramatic sensitivity to temperature, as signal transduction from the basolateral cell surface does not require movement to the contralateral apical membrane. After arrival

within Golgi cisternae or ER, the CT- G_{M1} complex internalized from the basolateral cell surface can move retrograde into the secretory pathway and back out again to adenylate cyclase on the basolateral membrane by remaining associated with membrane receptors derived from (and presumably targeted to) the basolateral membrane.

In summary, these studies identify Golgi cisternae and ER as intermediary compartments through which CT may traffic to elicit an efficient and maximal physiologic response. The unique lipid and protein structure of these compartments (3) may provide a thermodynamically or kinetically favorable environment for translocation of the A subunit. The COOH-terminal K(R)DEL sorting signal facilitates toxin entry into the secretory pathway, but movement into Golgi cisternae and possibly even ER will occur (though not efficiently) in the absence of the K(R)DEL-sorting signal after toxin binding (or clustering) ganglioside G_{M1} . Thus CT, as a paradigm for vesicular transport, provides an example where protein targeting (and ultimately the pathogenesis of cholera in humans) depends on the combined effects of protein and lipid structure in small membrane microdomains (58, 68).

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Note Added in Proof. After our manuscript was submitted, the following paper was published: Cieplak, W., Jr., R. J., Messer, M. E. Konkel, and C. C. R. Grant. 1995. *Mol. Microbiol.* 16:789–800. They also constructed and characterized an RDEV mutant of LT, but unlike the results in our study, concluded that K(R)DEL was not important for toxin trafficking.

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