Proteolytic Activation of Cholera Toxin and Escherichia coli Labile Toxin by Entry into Host Epithelial Cells

SIGNAL TRANSDUCTION BY A PROTEASE-RESISTANT TOXIN VARIANT

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Cholera and Escherichia coli heat-labile toxins (CT
and LT) require proteolysis of a peptide loop connecting
two major domains of their enzymatic A subunits for
maximal activity (termed “nicking”). To test whether host intestinal epithelial cells may supply the necessary
protease, recombinant rCT and rLT and a protease-resistant mutant CTR192H were prepared. Toxin activation
was assessed as a Cl−secretory response (Isc) elicited from monolayers of polarized human epithelial T84
cells. When applied to apical cell surfaces, wild type toxins elicited a brisk increase in Isc (80 μA/cm2). Isc was
reduced 2-fold, however, when toxins were applied to basolateral membranes. Pretreatment of wild type
with trypsin in vitro restored the “basolateral” secretory responses to “apical” levels. Toxin entry into
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restored the “basolateral” secretory responses to “apical” levels. Toxin entry into T84 cells via apical but not basolateral membranes led to
nicking of the A subunit by a serine-type protease. T84
cells, however, did not nick CTR192H, and the secretory response elicited by CTR192H remained attenuated
even when applied to apical membranes. Thus, T84 cells
express a serine-type protease(s) fully sufficient for ac-
tivating the A1 peptide to gain access to adenylate
ble for secretory diarrhea (10).

In nature, both toxins make initial contact with the intesti-
nal T84 cell line to examine the cell biology of Vibrio cholerae
and Escherichia coli heat-labile toxins on polarized epithelial
cells. Both toxins (cholera toxin, CT1, and labile toxin, LT) are
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cal (1–3). They account for the massive secretory diarrhea seen
in infections caused by these microorganisms (4–6).

CT and LT consist of five identical B subunits that bind
ganglioside GM1 at the cell surface and a single A subunit
comprised of two functional domains termed the A1 and A2
peptides (1, 2). The A1 peptide exhibits the toxin’s ADP-ribo-
syltransferase activity, which is necessary for signal transduc-
tion. The A2 peptide tethers the A and B subunits together and
contains the endoplasmic reticulum-targeting motif KR/DEL
at its COOH terminus. Enzymatic activity of the A subunit is
latent. For full ADP-ribosyltransferase activity, the peptide
loop connecting the A1 and A2 peptides must be proteolytically
cleaved at residue Arg-192 (7). After proteolytic cleavage
(termed “nicking”), the A1 and A2 peptides remain covalently
associated via a single disulfide bond. This bond is likely to be
reduced when the A1 peptide translocates across the membrane
to the cytosolic membrane surface (8). Translocation is neces-
sary for the A1 peptide to gain access to its substrate
the heterotrimeric GTPase Gαs (9). Toxin-induced ADP-ribosyla-
tion of Gαs activates adenylyl cyclase and raises intracellular
AMP levels, which in intestinal crypt epithelial cells elicits a
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ble for secretory diarrhea (10).

Over the last several years, we have used the human intesti-
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many Vibrios secrete their own proteases (17) and both CT and LT act within the gastrointestinal tract, it has largely been assumed that V. cholerae activates its own toxin, and proteases in the gut lumen activate LT. However, the very first events in the pathogenesis of diarrhea due to both V. cholerae and E. coli likely entail bacterial adhesion to the enteroocyte surface, as evidenced by the nature of identified invasion factors (type IV pilus, surface glycopolypeptides, and inner membrane regulatory proteins such as ToxR, (18, 19)). Thus, in vivo, both CT and LT may bind to the intestinal cell apical membrane immediately after release from the microbe.

Our aim in the present study was to examine whether the enteroocyte itself may proteolytically activate the nascent A subunits of cholera or E. coli heat-labile toxins. As before, we utilized the human intestinal cell line T84 to model the interaction between toxin and intestinal epithelial cell. Our results show that a serine protease(s) endogenous to the apical membrane or apical endocytic compartment of T84 cells is sufficient to activate fully nascent CT or LT. Proteolytic activation, however, is not apparent when toxin enters the cell via the basolateral CT at 4 °C for 30 min prior to shifting to fresh HBSS at 37 °C. Measurements of short circuit current and resistance were performed with 0.33-cm² monolayers, and biochemical studies were performed with 5-cm² monolayers as described previously (20–22).

Preparation of Recombinant Wild Type Toxins (rCT and rLT)—E. coli XL1-Blue M15 Tn10; (23) or TX1 harboring plasmids encoding wild (wt) CT or LT were inoculated from stocks into 200 ml of Luria-Bertani medium (L-broth) (24) 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 were 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 phosphate-buffered saline (140 mM NaCl, 10 mM NaHPO₄/Na₂HPO₄, pH 7.2) and then resuspended in 8 ml of ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM NaHPO₄/Na₂HPO₄, pH 7.2) and then resuspended in 8 ml of ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM NaHPO₄/Na₂HPO₄, pH 7.2) and then resuspended in 8 ml of ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM NaHPO₄/Na₂HPO₄, pH 7.2). Then 20 mL of HBSS at 37 °C was added to the cultures, and the mixture was incubated at 37 °C for 10 min. This extract was applied to a galactose depleted L-blot (26). Pooled fractions were then removed intact on their filter supports, immersed in 0.6 ml of 100 mM Tris, pH 7.4 containing 0.15 M NaCl, 0.2% Triton X-100, 1 mM PMSF, and 1 mM EDTA, and dialyzed extensively against HBSS and stored at −80 °C. Analysis of protein content in these pooled fractions by SDS-PAGE (reducing conditions) showed single protein bands at ~28 and 11 kDa (corresponding to A and B subunits) when stained by Coomassie Blue.

Assessment of Recombinant Toxin Concentrations—To assay toxin concentrations an enzyme-linked immunosorbent assay was used. Periplasmic extracts containing recombinant toxins were applied to 96-well microtiter plates coated with ganglioside GM₁ as described previously (27). After 30 or 60 min at 37 °C, the plates were washed with phosphate-buffered saline, pH 7.4, and the presence of CT or LT bound to GM₁ was assayed by routine techniques using rabbit polyclonal antiserum raised against either CT A subunit or CT B subunit (1,000 in phosphate-buffered saline) (11, 12) (both cross-react with LT subunits), or mouse monoclonal against LT B subunit (28). Apparent toxin concentrations were confirmed by SDS-PAGE and Western blot using serial dilutions of CT or LT B subunit as standards.

Tryptic Treatment of Recombinant CT and LT in Vitro—Periplasmic extracts containing final vials of recombinant toxin (0.1 mg/ml) were incubated with 0.2–2 mg/ml trypsin at 37 °C for 30 min as modified from methods described previously (7, 29, 30). The reaction was stopped by adding 200 mg/ml soybean trypsin inhibitor at 4 °C. Nicking was assessed structurally by SDS-PAGE and Western blot and functionally by increase in efficiency of toxin-induced Clᵢ secretion after applying the nicked toxin to basolateral reservoirs of T84 cell monolayers. Nicking damage to the recombinant toxin was assayed for functional activity as a reduction in potency of toxin-induced Clᵢ secretion after applying the nicked toxin to apical reservoirs of T84 cell monolayers.

Proteolytic Nicking of rCT and CTR192H by T84 Cells in Situ—Monolayers were incubated with acipal or basolateral rCT or CTR192H (20 ng) at 4 °C for 15 min before transferring to fresh buffer at 37 °C or at 4 °C for the indicated times (up to 180 min). Nicking of the CT A subunit was assessed as a 5-kDa shift in molecular mass after immunoprecipitation, SDS-PAGE, and Western blot as described below.

To identify the “class” of protease likely responsible for nicking, we utilized inhibitors of the proteolytic reactions catalyzed as described in (31). Monolayers were preincubated with the specified protease inhibitors or buffer alone (containing the carrier dimethyl sulfoxide, Me₂SO) at 4 °C for 20 min before rCT or CTR192H (20 ng) was added. The following protease inhibitors were used to determine “functionally” the class of protease involved (based on reaction catalyzed). Serine-type peptidases were diisopropyl fluorophosphate (DFP, 2.5 mM), phenylmethylsulfonyl fluoride (PMSF, 175 μM from 35 mM stock in ethanol), and 3,4-dichloroisocoumarin (3,4-DCI, 1 mM from 100 mM stock in Me₂SO). Cysteine-type peptidases were leupeptin (1 μM, from 1 mM stock in water) and E-64 (0.2 μM stock in water). Metallopeptidases were EDTA (5 mM), and 1,10-phenanthroline (1 mM from 100 mM stock in Me₂SO). The aspartic-type peptidase was pepstatin (1 mM from 100 mM stock in Me₂SO).

Immunoprecipitation, SDS-PAGE, and Western Blotting—Monolayers were incubated with acipal or basolateral rCT or CTR192H (20 ng) at 4 °C for 15 min before transferring to 37 °C for the indicated times (to allow endocytosis and entry of toxin into the cell). Control monolayers were kept at 4 °C for the duration of the experiment (90 or 120 min). Little or no endocytosis occurs at 4 °C. After incubations at 37 °C were then removed intact on their filter supports, immersed in 0.6 ml of 0.5% SDS, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 20 mM triethanolamine, 0.18 mM PMSF, and 20 μg/ml chymostatin, and heated at 100 °C for 3 min. The cell lysate was diluted with Triton X-100 to form a mixed micelle buffer (0.25% SDS, 2% Triton X-100, final). DNA was sheared by vortex at 4 °C for 30 min and removed by centrifugation (12,000 x g for 10 min) in the presence of Sepharose CL-4 (Pharmacia Biotech Inc.) until clear. Lysates were precentrifuged again by a 30-min incubation with A-Sepharose. This provided cellular proteins and proteins associated with the cell monolayer including the entire fraction of toxin bound to the cell surface or internalized via endocytosis (11, 12, 25).

After cell lysis and removal of all cellular and cell-associated proteins, CT A and B subunits were immunoprecipitated using polyclonal antibodies raised against denatured toxin subunits eluted from
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RESULTS
Proteolytic Activation of rLT and rCT by Host Epithelia: Role of Cell Polarity—In recent studies utilizing recombinant CT and LT (25), we noticed that the time courses of Cl− secretion elicited by basolaterally applied toxins (20 nm) were slower and significantly less robust than we had previously observed with toxin preparations purified from V. cholerae supernatants. In contrast, when these same recombinant toxins were applied to apical membranes, there was little or no delay in the anticipated time course of the secretory response. In all past experiments (11, 12, 21, 25, 32), we consistently found that toxin preparations purified from V. cholerae supernatants elicited Cl− secretion with a shorter lag phase and more rapid rate of onset when applied to basolateral rather than apical surfaces of the same cells (Fig. 1, panel A). Unmistakably, the faster rate of signal transduction elicited by recombinant toxins (rCT or rLT) entering the cell through the apical endosome was altogether opposite to that observed previously in earlier studies (Fig. 1, panels B and C). As preparations of native CT purified from V. cholerae supernatants are proteolytically nicked and preparations of recombinant toxins expressed in E. coli XL1-Blue are not, we hypothesized that when applied to basolateral surfaces of T84 cell monolayers the attenuated time courses of signal transduction elicited by recombinant rCT or rLT were due to the absence of or a delay in A subunit activation. In vitro, the enzymatic activities of both CT and LT are enhanced 10-fold by proteolytic cleavage (activation) of the A subunit (7).

To test this idea, we proteolytically nicked recombinant rCT and rLT in vitro by pretreatment with low dose trypsin. Pretreatment with trypsin resulted in nicking of >20% of total A subunit (as assessed by SDS-PAGE) without attenuating ADP-ribosyltransferase activity (as assessed by the ability of nicked toxins to elicit a secretory response when applied to apical surfaces of T84 cells). Fig. 2 shows the time course of Cl− secretion elicited by recombinant rCT (20 nm) and rLT (40 nm), nicked or not nicked by pretreatment with trypsin in vitro. When applied apically, toxins not pretreated with trypsin elicited a brisk secretory response from T84 cells (upper panels). When applied to basolateral membranes, however, the secretory responses elicited by these recombinant toxins were clearly attenuated (lower panels). Signal transduction by toxin entering the cell through the basolateral membrane displayed a longer lag phase and slower rate of onset compared with the time courses of Cl− secretion elicited by the same toxin preparations applied apically. Pretreatment of rCT or rLT with trypsin in vitro, however, resulted in a faster time course of signal transduction elicited by the basolaterally applied toxins, presumably due to nicking of the A subunit (upper panels, +Trypsin). Trypsin had no effect on the time course of apically applied toxins (upper panels, +Trypsin). These data (summarized in Table I) show that proteolytic activation of CT or LT is rate-limiting for signal transduction when toxin enters the cell via the basolateral (but not apical) cell surface. As such, these results suggest that entry of nascent CT or LT into the apical endosome leads to cleavage of the A subunit at position Arg-192, but this does not occur when toxin enters the cell via the basolateral endocytic pathway.

Nicking of CT A subunit occurs during toxin entry via the Apical but Not Basolateral Cell Surface—To examine whether T84 cells could activate nascent CT by proteolytic nicking of the A subunit, we again utilized recombinant toxins. rCT was applied to apical (Fig. 3, upper panel) or basolateral (Fig. 3, lower panel) reservoirs of T84 monolayers at 4 °C and then shifted to 37 °C or kept at 4 °C for the indicated times. The entire fraction of toxin associated with the monolayers was then solubilized by lysing the monolayers in 0.5% SDS at 100 °C as described under “Experimental Procedures.” Toxin subunits were concentrated by immunoprecipitation, and nick-
ing was assessed as a shift in apparent molecular mass of the A subunit from 28 to 22 kDa by SDS-PAGE and Western blot. Fig. 3 shows the results of these studies. The first lane from the left in the upper panel shows that when toxin was applied apically, incubations at 4 °C led to proteolytic nicking of the A subunit. At 37 °C (third through seventh lanes in the upper panel), nicking was apparent by 15 min and progressed steadily over time. When applied to basolateral cell surfaces, however, nicking of the A subunit was not apparent even after a 2-h incubation (lower panel). Proteolysis of as little as 0.5% of total toxin bound to T84 cell monolayers can be detected by Western blot (assessed by serial dilution’s of toxin bound at 4 °C). Thus, toxin entry into the cell via apical endosomes resulted in cleavage of the A subunit into A1 and A2 peptides. Nicking was time-dependent and appeared able to occur at the cell surface as evidenced by proteolysis of toxin bound to apical cell surface receptors at 4 °C.

To determine the class of protease responsible for this cleavage (as defined by reaction catalyzed; 31), we preincubated T84 cell monolayers with serine, cysteine, aspartic, and metalloprotease inhibitors before applying recombinant CT. As shown in Fig. 4, A and B, cleavage of rCT A subunits was inhibited completely by the serine protease inhibitors DFP (2.5 μM), and 3,4-DCI (1 μM). PMSF (175 μM) blocked cleavage of the rA subunit strongly but incompletely. Nicking was not inhibited by the metalloprotease inhibitors EDTA (5 mM) and 1,10-phe-

**TABLE I**

Effect of trypsin treatment on kinetics of Cl⁻ secretion elicited by recombinant LT or CT entering the cell via apical or basolateral cell surfaces

The Cl⁻ secretory response was assessed as a short circuit current (Isc). In vitro activation of either recombinant toxin by trypsin treatment resulted in a significant acceleration of signal transduction when CT or LT entered the cell from the basolateral but not apical membrane. Mean ± S.E. N represents the number of independent experiments with each experiment comprised of two independent samples/group. For wt rLT and nicked rLT applied apically, calculated means were based on N = 3 and N = 2, respectively. For wt rLT and nicked rLT applied basolaterally, calculated means were based on N = 5 and N = 3, respectively.

| Toxin  | Lag phase | dIsc/dt | Peak Isc | Lag phase | dIsc/dt | Peak Isc |
|--------|-----------|---------|----------|-----------|---------|----------|
|        | min       | μA/cm²/min | μA/cm² | min       | μA/cm²/min | μA/cm² |
| wt LT  | 46 ± 2    | 0.5 ± 0.1 | 39 ± 3   | 51 ± 3    | 0.33 ± 0.08 | 38 ± 4  |
| Apical |           |         |          |           |         |          |
| N = 2–3|           |         |          |           |         |          |
| Basolateral | 66 ± 3    | 0.23 ± 0.07 | 26 ± 6  | 42 ± 3    | 0.75 ± 0.05 | 55 ± 6  |
| wt CT  | 45 ± 2    | 1.5 ± 0.2 | 78 ± 0.7 | 45 ± 2    | 1.6 ± 0.5  | 82 ± 8  |
| Apical |           |         |          |           |         |          |
| N = 3  |           |         |          |           |         |          |
| Basolateral | 61 ± 6    | 0.8 ± 0.3 | 49 ± 16  | 37 ± 7    | 2.6 ± 0.9 | 83 ± 12 |

* Statistically significant differences between parameters describing the time course of Cl⁻ secretion induced by recombinant toxins nicked or not nicked in vitro with trypsin as assessed by ANOVA (p = 0.0001).
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Fig. 3. Western blot of immunoprecipitated A subunit after incubating CT with apical or basolateral membranes of T84 cell monolayers. Two-point calibration was obtained by applying CT at 4 °C (120 min) to either apical (Ap, first lane from left) or basolateral cell surfaces (B, second lane). The third through seventh lanes show the time course of nicking at 37 °C after applying CT to apical (upper panel) or basolateral membranes (lower panel). Data shown are representative of three separate studies.

Fig. 4. Effect of protease inhibitors on the ability of T84 cell monolayers to nick CT. Panel A, T84 cells were preincubated at 4 °C for 15 min with 2.5 μM DFP, second, fourth, and fifth lanes from left), EDTA (5 mM, sixth lane), leupeptin (1 μM, seventh lane), or no protease inhibitor (first lane). Recombinant wt CT (20 nM) was then applied to apical cell surfaces and incubated for a further 180 min. Some monolayers were shifted to 37 °C where indicated. Each lane represents an independent experiment. Data shown are representative of two separate studies. Panel B, T84 cells were preincubated at 4 °C for 30 min with HBSS containing 1% Me_2SO (DMEM, lane 2), PMSF (175 μM, lane 3), pepstatin (1 μM from 100 mM in Me_2SO, lane 4), E-64 (10 μM, lane 5), 1,10-phenanthroline (1 mM, lane 6), or 3,4-DCI (1 mM, lane 7). Recombinant wt CT (20 nM) was then applied to apical cell surfaces and incubated for a further 120 min at 4 °C. Lane 1 shows the positions of the A subunit and A1 peptide from nicked wt CT purified from Vibrio

entry into T84 cells via the basolateral endocytic pathway did not result in detectable nicking of the A subunit (Fig. 3, lower panel). Nevertheless, recombinant toxins applied to basolateral cell surfaces elicited an attenuated but clearly detectable secretory response (Figs. 1 and 2).

Protease-resistant CT Variant—To confirm our interpretation of these results, we prepared recombinant rCT replacing Arg-192 with histidine (CTR192H). This mutation inactivates the trypsin nicking site connecting the A1 and A2 peptides of the A subunit. Fig. 5 shows that CTR192H was not nicked by T84 cells, even after 2-h incubation at 37 °C. CTR192H, however, was still able to elicit a secretory response (Fig. 6). When applied basolaterally, the secretory response elicited by CTR192H was similar to that of wt but not nicked rCT (Fig. 6, lower panel, and Table II). In contrast, when applied to apical cell surfaces, the time course of Cl⁻ secretion elicited by wt rCT was accelerated (presumably due to nicking), but the response elicited by mutant CTR192H remained dramatically attenuated (Fig. 6, upper panel, and Table II). These data confirm our findings that intestinal T84 epithelial cells proteolytically activate nascent rCT when the toxin enters the cell through the physiologically relevant apical membrane.

nананолине (1 mM) or by the cysteine peptidase inhibitor leupeptin (1 μM). The aspartic-type protease inhibitor pepstatin (1 μg/ml) and the cysteine peptidase inhibitor E-64 (1 μM) displayed incomplete inhibition. Both pepstatin and E-64 may interfere with reactions catalyzed by proteases of other classes, notably those that may exhibit significant thiol dependence or those maximally active at low pH and dependent on carboxyl groups (31).

When taken together, these data provide evidence that the nascent A subunits of CT and LT are likely activated by a serine protease(s) endogenous to the apical membrane or apical endosome, or both, of human intestinal T84 cells. In contrast,

DISCUSSION

The results of these studies show that nascent rCT (and presumably rLT) can be proteolytically activated by a serine protease(s) endogenous to the apical membrane or apical endosome (or both) of human intestinal T84 cells. In contrast, toxin entry into the cell via basolateral endosomes did not result in nicking of either rCT or rLT A subunit, and this appears to be rate-limiting for signal transduction. These data provide evidence that in vivo, after colonization of the intestine by V. cholerae or E. coli, the process of toxin binding and entry into the host intestinal cell via the physiologically relevant apical membrane may be fully sufficient for activation of the nascent enterotoxins secreted by these microbes.

Although full activity of both CT and LT requires proteolytic cleavage of the exposed loop connecting the A1 and A2 fragments of their respective A subunits (residues 187–199), the amino acid sequences of these functionally conserved regions lack any real sequence homology, with the exception of Arg at position 192 (1, 33). The importance of this residue in toxin action is confirmed by replacing Arg-192 with His in recombinant toxin CTR192H. Neither trypsin treatment in vitro nor proteases endogenous to T84 cells in situ were able to cleave the A subunit of CTR192H into A1 and A2 fragments, and this was correlated with a clear decrease in the ability of mutant CTR192H to induce Cl⁻ secretion. Similar results confirming the importance of Arg-192 in LT were obtained by replacing
Arg-192 with Gly as described recently by Grant et al. (34) and Dickinson and Clements (35).

The functionally defined T84 cell protease(s) responsible for cleavage of the A subunit displayed complete sensitivity to both DFP and 3,4-DCI and likely represents a membrane-associated serine protease (31). E-64 (1 mM) also inhibited nicking, though not completely. The related cysteine peptidase inhibitor leupeptin, however, did not. The aspartic-type protease inhibitor pepstatin appeared to have an effect on nicking. As such, these data provide some evidence that the apically located serine- type protease(s) responsible for toxin activation may exhibit significant thiol or pH dependence, or both (such as that found for the carboxypeptidase C family, prolyl oligopeptidase, and a subset of the subtilisin family) (31).

The molecular identity of the protease responsible for nicking the CT A subunit remains unknown. Although intestinal epithelia in vivo express at least two endoproteases and numerous peptidases on their luminal membranes (36), the activities of cell surface or endosomal proteases in human T84 cells have not been systematically examined. T84 cells do express the serine protease tissue-kallikrein (37, 38), together with a novel serpin protease inhibitor kalistatin (39). These data raise the possibility that T84 monolayers may activate nascent CT and LT by utilizing tissue-kallikrein. T84 cells also express furin, as evidenced by the ability of T84 cells to activate, process, and respond to edema factor/protective antigen of nascent anthrax toxin.3 Nascent anthrax toxin must be cleaved by furin to elicit a response from intact cells. However, the expression of furin by T84 cells cannot explain our results as neither CT nor LT contains the RXRX amino acid motif required for furin cleavage (16). Whatever the molecular identity, this functionally defined protease must display a remarkable degree of apical polarization as little or no activity can be detected when CT or LT enters the cell from basolateral membranes.

The results of these studies also show that proteolytic nicking of the A subunit may not be necessary for toxin action, as both CTR192H and wt but un-nicked rCT (i.e. basolaterally applied) elicit a Cl- secretory response from T84 monolayers. Similar findings were reported by Grant and co-workers for a nearly identical mutation in LT tested on Chinese hamster ovary and nonpolarized Caco-2 cells (34). It has also been well described that the intact A subunit displays clear (though attenuated) enzymatic activity in vitro (7). Nevertheless, it remains possible that after entry into basolateral or apical endosomes, both wt and mutant CTR192H may be nicked at position 192 or at an alternative site(s), in very small amounts not detectable by Western blot, by the same or another protease. When taken together, however, these data provide evidence that in vivo protease-resistant variants such as CTR192H may continue to display the ability to elicit secretory diarrhea though with attenuated potency.

Since the A subunits of both CT and LT maintain extensive and stable noncovalent interactions with the B pentamer (2, 7), the ability of un-nicked CTR192H to elicit a Cl- secretory response raises the distinct possibility that domain A1 of CTR192H may translocate across the membrane and exhibit enzymatic activity in the cytosol while tethered via the A2 domain to the B subunit on the contralateral (lumenal) membrane surface. If this is correct, it is also possible that the A1 peptide of nicked CT or LT may not fully dissociate from the A2 peptide and B subunit after entry into the cell. If so, this would fit nicely with our previous observations that in polarized cells signal transduction by CT is not diffusion-limited even after translocation of the A subunit (21) and that both the pentameric B subunit and translocated A subunit appear to travel together across the cell en route to the basolateral membrane (11). Alternatively, it remains possible that the A1 peptide of both mutant and wt toxin may dissociate from the A2 peptide/B subunit-GM1 complex after a small (but not measurable) amount of nicking by the same or another protease as discussed above or that proteolytic cleavage of the A subunit is not required for complete dissociation of A subunit from the B pentamer. In support of this possibility, the entire CT A subunit including the A2 peptide appears to separate from the pentameric B subunit after toxin entry into Vero cells (13, 14).

In summary, these studies show that in nature the host intestinal cell may engage V. cholerae or E. coli in a form of molecular interaction by supplying the necessary protease to

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3 W. I. Lencer and R. J. Collier, unpublished results.
activate the nascent A subunits of cholera or E. coli heat-labile toxins. As modeled by the T84 cell system, a protease(s) endogenous to the enterocyte apical membrane, or endosome, or both is fully sufficient to nick the A subunits of CT and LT after they are released from the microbe and bind to the cell surface. Soluble proteases produced by V. cholerae or endogenous to the gut lumen may have little or no effect on the activation of these enterotoxins in vivo.

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