The severe, and at times fatal, diarrheal disease caused by Vibrio cholerae is due to the potent action of cholera toxin (Ctx)\(^1\) (for a review, see Ref. 1). A structurally and functionally similar toxin, heat-labile enterotoxin (Etx), is produced by certain strains of enterotoxigenic Escherichia coli that are responsible for causing the generally milder “traveler’s” diarrhea of humans and scouring in farm animals (2, 3). Both Ctx and Etx are hetero-oligomeric proteins comprised of a single A-subunit (\(M_r 28,000\)) and five B-subunits (\(M_r 12,000\) each) (4–6). The A-subunit contains two distinct structural domains linked by a disulfide bridge: an A1-fragment (residues 1–192) that displays ADP-ribosyl transferase activity and an A2-fragment (residues 193–240) that mediates interaction with the B-subunit pentamer (4, 6). The B-subunits of Ctx and Etx (CtxB and EtxB, respectively) bind to cell surface receptors, principally \(G_{M1}\)-ganglioside, found ubiquitously on the plasma membranes of eukaryotic cells (7). Although Ctx and Etx exhibit a remarkable degree of structural homology, with 81.6\% sequence identity between CtxA and EtxA and 82.5\% sequence identity between CtxB and EtxB (8–10), there are a number of subtle physicochemical and functional differences between the two toxins. For example, although the B-subunit pentamers of both toxins are resistant to environmental conditions that normally lead to protein denaturation, the pH stability of EtxB is almost 2 orders of magnitude greater than that of CtxB (11). Studies have also shown that the receptor binding specificities CtxB and EtxB are slightly different; whereas both bind with high affinity to ganglioside \(G_{M1}\), and to a lesser extent to \(G_{D1h}\), EtxB also binds significantly to glycoprotein receptors and polyglycosylceramides, and with lower affinity to \(G_{M2}\), asialo-\(G_{M1}\), and paragloboside (12–17). Sequence differences between CtxA and EtxA are spread throughout the polypeptides with the lowest identity surrounding the A1/A2 cleavage site and near the C terminus of the A2-fragment (10). The influence on toxin function of most of the primary amino acid sequence divergence has not yet been fully explored.

The action of cholera toxin and related enterotoxins on eukaryotic cells depends on a complex sequence of events that eventually leads to alterations in ion fluxes and a concomitant loss of water, characteristic of cholera and related diarrheal diseases. The use of polarized human colonic epithelial T84...
cells has greatly facilitated studies of Ctx and Etx, since toxicity can be readily monitored as the induction of electrogenic Cl\(^-\) secretion (18). Toxin action is initiated by binding of the B-subunit moiety to cell surface receptors. Recently, it was demonstrated that, following binding of Ctx to T84 cells, Ctx-G\(_{M3}\) complexes cluster in caveolae-like detergent insoluble subdomains of the plasma membrane (19). Invagination and internalization of these membrane domains results in the formation of smooth endocytic vesicles that enter vesicular trafficking pathways leading to transport of the toxin to the trans-Golgi network (TGN) (18, 20–25). The observation that brefeldin A inhibits cholera toxin action (20, 21, 26), and the presence of a KDEL sequence at that the C terminus of CtxA (RDEL in EtxA) have suggested that the toxin is transported from the TGN to the endoplasmic reticulum (ER). Indeed, mutations in the K/RKDEL sequence of CtxA and EtxA reduce the efficiency of toxin-induced Cl\(^-\) secretion in T84 cells (22). It has been speculated that the A-subunit may detach from the B-subunits in the TGN, since only the A-subunit (A1/A2-fragments) have been detected in the ER (23–25), although transport of the holotoxin from the Golgi to the ER, followed by rapid dissociation and anterograde transport of the B-subunit back to the Golgi, has not been excluded. Reduction of the disulfide bond between the A1- and A2-fragments is thought to be catalyzed by protein disulfide isomerase resident in the ER (27, 28), followed by translocation of the A1-fragment across the ER membrane to the cytosolic compartment. Because of its hydrophobicity, the A1-fragment may remain associated with the cytosolic face of the ER membrane, rather than being released free into the cytosol. The subsequent trafficking and presumed delivery of the A1-fragment to the basolateral membrane are less well understood, although the finding that the A-subunit interacts with “so-called” ADP-ribosylation factors (ARFs) (29), involved in vesicular transport, may facilitate its anterograde targeting to the basolateral membrane. ARFs have also been demonstrated directly to increase toxin activity in vitro (30), a finding that may be important in determining the magnitude of toxin action in vivo. The A1-fragments of Ctx and Etx accomplish their toxic effects by ADP-ribosylating G\(_{M3}\), a component of the trimeric GTP-binding protein that activates adenylyl cyclase.

In T84 cells, toxin-induced elevations in cAMP levels lead to electrogenic Cl\(^-\) secretion: the primary ion transport event responsible for secretory diarrhea in humans.

For Ctx and Etx to exhibit full toxicity, the A-subunits must undergo proteolytic cleavage or “nicking” at Arg-192 to give separate A1- and A2-fragments (31). In the case of cholera toxin, extracellular proteases, such as HA protease produced by V. cholerae, can efficiently nick and activate the A2-subunit (32). By contrast, Etx from enterotoxigenic E. coli, as well as recombinant Ctx produced in E. coli, are normally isolated with their A-subunits intact; trypsin or other gut-associated proteases have been postulated to accomplish toxin activation in such cases (33). Recent studies on T84 cells have shown that a serine protease, which efficiently nicks and activates both CtxA and EtxA, is present on either the apical surface or in apically-derivated transport vesicles (31). In this respect, no difference was found in toxicity of commercial (nicked) preparations of Ctx and recombinant Ctx that was either unnicked or nicked with trypsin.

The time course and magnitude of electrogenic Cl\(^-\) secretion in T84 cells demonstrated that Ctx (hereafter referred to as CtxAB) was significantly more active than Etx (hereafter referred to as EtxAB); CtxAB exhibited a shorter apparent lag period and generated a higher short circuit current (22). In this paper, we describe the structural basis for this difference in toxicity. The data reveal that it is not due to structural or functional differences in the ADP-ribosylating A1-fragment or in the receptor-binding B-subunits, but to the A2-adapter fragment, which mediates A/B-subunit interactions. These findings highlight the importance of a region in the toxin that has hitherto been generally overlooked, and provides a possible contributory explanation for the difference in severity of cholera and traveler’s diarrheal disease.

**EXPERIMENTAL PROCEDURES**

**Materials**

All reagents were purchased from Sigma or BDH unless otherwise stated. Monoclonal antibodies CT-17, which recognizes CtxA was the gift of Professor J. Holmgren (University of Gothenburg, Gothenburg, Sweden), and 118-8, which recognizes the CtxB and EtxB, was provided by Dr. H. Person (University of Umeå, Umeå, Sweden). Recombinant human ADP-ribosylation factor-6 (rhARF 6) was provided by Dr. W. Patton (NHLBI, National Institutes of Health, Bethesda, MD)

**ctx and etx Operons**

The ctxAB operon encoding the A- and B-subunits of cholera toxin was amplified by PCR from the template plasmid, pBK33 (34) using the following primers: 5′-TTGGGCCGATATTCCTTTGTAAACAAA-G-3′ (CROL1) and 5′-GCTCTAGACTGTTGGCCATACATAATTCCGGC-C-3′ (CROL2) and Expand\(^{TM}\) high fidelity polymerase (Boehringer Mannheim). The resulting fragment was cleaved with ApaI and SpeI and then ligated into pBluescript II KS\(^+\) (Stratagene) that had been cut with the same restriction enzymes to yield pRC1 (Fig. 1). To facilitate control of each level expressed, the ctxAB operon from pRC1 was subcloned into pTQ78 (35) at the Small and Xbal sites to yield pRC9. Plasmid pTRH29 was described previously and is a pBluescript derivative containing the etxAB operon of E. coli enterotoxin flanked by EcoRV and SpeI restriction sites (36). A pTQ78 derivative, harboring the EcoRV-SpeI fragment from pTRH29 and designated pMAM9, has been described (37).

**Construction of Mutant and Hybrid Toxin Operons**

Mutant and hybrid operons were constructed by ligating two separate fragments derived by PCR amplification of upstream and downstream segments of the ctxAB or etxAB operons.

**Construction of a Hybrid operon Encoding CtxA(1–224)-CtxB**

pB393 was used as the template for amplification of a 5′-fragment encoding CtxA using primers CROL1 and 5′-TCATCCGGATTCTTATAT-GTG-3′, and a 3′-fragment encoding CtxB using primers 5′-ATA-GAATTCCGGATCAGA-3′ and CROL2. The 3′-fragment was cloned into pBluescript at the EcoRI-SpeI sites, yielding pATAS. This plasmid was subsequently cut with ApaI and EcoRI, and the 5′-PCR fragment inserted to reconstruct the ctxAB operon, yielding plasmid pATA14 (Fig. 1). The EcoRI site in pATA14 at the junction between the genes encoding the A- and B-subunit resulted in replacement of Lys-237 by Arg in CtxA. The EcoRV-SpeI fragment of pATA14 was subcloned into pTQ78, yielding pCDR3.

**Construction of a Hybrid operon Encoding EtxA(1–224)-CtxA**

CtxB-pTRH29 was used as the template for amplification of a 5′-fragment from the ctxA gene using the universal reverse primer (Stratagene) 5′-AACACGTAGCACCAGT-3′ and oligonucleotide 5′-CTCTGACTGGATCCCTGAA-3′. The DNA fragment was cleaved with EcoRV and KpnI and inserted into these sites in pBluescript to yield pRC7. pRC1 was used as the template for amplification of a 3′-fragment of the ctxAB operon from codon 226 of ctxA using the oligonucleotide 5′-TTTCCGGATCAGCTGACG-3′ and the 3′-primer (Stratagene) 5′-TTTCTCCGACATGCGAC-3′. The fragment was then cloned with KpnI and SpeI and inserted into the corresponding sites of pBluescript, yielding pRC2. The hybrid operon was constructed using an intermediate vector pRCK18, a derivative of pK18 (38) containing the KpnI-StuI fragment of the hybrid operon encoding the A- and B-subunits of cholera toxin. The resultant plasmid was cleaved with EcoRV and SpeI and the hybrid operon cloned into the correspond-
Differential Toxicity of Cholera Toxin and E. coli Enterotoxin

**Fig. 1. Wild-type and hybrid toxins.** Plasmid pRC1 contains the entire ctxAB operon encoding wild-type CtxA; plasmid pTRH29 (36) contains the entire etxAB operon encoding wild-type EtxA; plasmid pATA14 encoding CtxA(1–225)EtxB was generated by PCR amplification of upstream and downstream segments of the ctxAB operon followed by their ligation at an engineered EcoRI site to introduce a Lys-237 to Arg substitution; plasmid pRC18 encoding EtxA(226–240)ctxB was generated by ligating an upstream segment of etxA to a downstream segment of the ctxAB operon at an engineered KpnI site; pRC19 encoding CtxA(1–225)etxAB was generated by ligating an upstream segment of ctxA to a downstream segment of the etxAB operon at an engineered KpnI site; and pCDRI encoding CtxA(1–225)etxA(226–240)–EtxB was constructed by substituting the EcoRI–KpnI fragment in pTRH29 for the corresponding fragment from pATA14. In all cases, the vector pBlue-script II KS is not depicted.

**Construction of a Hybrid Operon Encoding CtxA(1–225)etxB—etxAB.**—The hybrid operon was constructed in a similar manner to that in pRC18. The 5′-fragment from the ctxA gene was amplified from pRC1 using the universal reverse primer and oligonucleotide 5′-GATGTTGATCCCTGAAA-3′, and the 3′-fragment of the etxA operon from codon 226 of etxA was amplified from pTRH29 using the oligonucleotide 5′-TTTCAAGGTTACGACTGCA-3′ and the −40 primer. These were cloned separately into pBlueScript to yield pRC3 and pRC6, respectively. The subsequent subcloning of these fragments to generate pRC1 using the universal reverse primer and oligonucleotide 5′-GATGTTGATCCCTGAAA-3′, and the 3′-fragment of thectxA operon was carried out in a manner identical to that described above for the construction of pRC18. The resultant plasmid encoding CtxA(1–225)etxA(226–240)–etxB was designated pRC19 (Fig. 1). The EcoRI–SpeI fragment of pRC19 was subcloned into pTTQ18, yielding pRC21.

**Construction of a Hybrid Operon Encoding CtxA(RDEL)etxB—etxAB.**—The EcoRI–EcoRI fragment from pTRH29 was substituted for the same fragment derived from pATA14, yielding plasmid pCDRI (Fig. 1). The EcoRI–SpeI fragment of pCDRI was subcloned into pTTQ18, yielding pCDRI2.

The DNA sequences of all of the cloned operons and constructs were verified by dyeoxy nucleotide sequencing.

**Toxin Assays**

**Electrophysiology—**T84 cells (from passages 75–100) obtained from ATCC were grown and passaged as described (18, 41). Toxins were diluted in prewarmed Hanks′ balanced salt solution (HBSS) containing (per liter): 0.185 g of CaCl2, 0.098 g of MgSO4, 0.4 g of KCl, 0.06 g of KH2PO4, 8 g of NaCl, 0.048 g of Na2HPO4, 1.0 g of glucose, and 10 mM Hepes at pH 7.4 and applied to the apical surface of confluent T84 cell monolayers in Transwell inserts (Costar, Cambridge, MA), followed by incubation at 37 °C. Alternatively, T84 cells were placed at 4 °C; the toxins diluted in ice-cold HBSS were then applied apically for 30 min at 4 °C. Cells were washed to remove unbound toxin, fresh HBSS was added, and the cells were incubated at 37 °C. Measurements of short circuit current (Isc) and resistance (R) were performed as reported elsewhere (18, 41).

**Toxin-catalyzed ADP-ribosylation of Agmatine—**To assay NAD:agmatine ADP-ribosyltransferase (30), the reaction was initiated by the addition of 1 μg of toxin to a mixture (total volume 0.3 ml) containing 10 mM Hepes, 50 mM potassium phosphate (pH 7.5), 0.5 mM unlabeled U-14C)NAD (90,000 cpm), 20 mM dithiothreitol, 5 mM MgCl2, 100 μM GTP, ovalbumin (0.1 mg/ml), 3 mM dimethylsphosphatidylcholine, and 0.2% cholate, with or without 1 μM rat ARF 6. After incubation for 1 h at 30 °C, duplicate 0.1-ml samples were transferred to columns (0.4 × 5 cm) of AG-1-X2 (Bio-Rad) followed by five washes with 1.0 ml of water, 30 °C, duplicate 0.1-ml samples were transferred to columns (0.4 × 5 cm) of AG-1-X2 (Bio-Rad) followed by five washes with 1.0 ml of water, which were collected for radioassay in a liquid scintillation counter (30).

**Assessment of Holotoxin Stability**

The stability of A- and B-subunit interaction in hybrid toxins was determined using an adaptation of a GM1-based enzyme-linked immunosorbent assay (42). 96-well microtiter plates were coated with GM1-ganglioside (1.5 μg/ml) for 24 h at 4 °C and then washed with PBS. The plates were incubated with 1% (v/v) BSA in PBS for 30 min at 37 °C and then washed with PBS, 0.1 ml of toxin (1.5 μg/ml) in PBS with 0.1% (v/v) BSA was then added to each well. After 1 h at room temperature, the plates were washed three times with PBS containing 0.5% (v/v) Tween 20, and once with PBS, and then the effect of various treatments on the stability of GM1-bound toxin was assessed. PBS alone, PBS containing 0.5% SDS, or McIlvaine buffer, pH 5.5, containing 0.5% SDS (0.15 ml) was added for 30 min at room temperature. Plates were then washed with PBS containing 0.05% (v/v) Tween 20, followed by the addition of either an anti-CtxA monoclonal antibody (CT-17) or an anti-EtxB monoclonal antibody (CT-18).
EtxAB (100 nM, nicked) was tested on T84 cells the toxins rate-determining step defining the magnitude and time course. It further demonstrates that nicking activate the A-subunits of cholera toxin and T84 cells (Fig. 3). This is consistent with our previous finding of the corresponding to intact (unnicked) A-subunits (A), the nicked A1-fragment (A1), and B-subunit monomers (B) are indicated. The position of the molecular weight markers are indicated on the right-hand side of the figure.

anti-EtxB monoclonal antibody (118-8) (43) in PBS containing 0.1% BSA with 0.05% Tween 20. After 1 h at room temperature, plates were washed with PBS containing 0.05% Tween 20, followed by the addition of goat anti-mouse IgG-horseradish peroxidase conjugate for 1 h at 37 °C. The plates were then washed with PBS containing 0.05% Tween 20 and developed with 0.1 ml/well 1 mg/ml 3% H2O2-phenylenediamine in 0.1 M citrate buffer, pH 4.5, containing 0.4 μl/ml 30% H2O2. Absorbance was measured at 450 nm in a microtiter plate reader (Anthos).

RESULTS AND DISCUSSION

Ctx and Etx Exhibit Differential Toxicity in T84 Monolayers—Previously, when samples of periplasmic fractions from E. coli that contained equivalent amounts of CtxAB or EtxAB were applied to the apical surfaces of polarized T84 cells, the time course of Cl− efflux elicited by the toxins were markedly different; a higher rate of Cl− secretion was induced by CtxAB than by EtxAB (22). This suggested that structural or functional attributes in either the A- or B-subunits of cholera toxin confered enhanced toxicity. To investigate this further, highly purified CtxAB and EtxAB were prepared as described under “Experimental Procedures.” Briefly, recombinant plasmids encoding CtxAB or EtxAB were introduced into the non-toxigenic V. cholerae O395NT strain, and the toxins purified from the culture media. The CtxAB preparation eluted as a single homogeneous peak on anion-exchange chromatography. When the culture media containing the reducing agent dithiothreitol prior to analysis by SDS-polyacrylamide gel electrophoresis.

FIG. 2. Purification of homogeneous preparations of “nicked” and “unnicked” toxins. CtxAB and EtxAB were purified as described under “Experimental Procedures,” and boiled in SDS sample buffer containing the reducing agent dithiothreitol prior to analysis by SDS-polyacrylamide gel electrophoresis. Lane 1, CtxAB (nick); lane 2, EtxAB (nick); lane 3, EtxAB (nicked). The migration positions corresponding to intact (unnicked) A-subunits (A), the nicked A1-fragment (A1), and B-subunit monomers (B) are indicated. The position of the molecular weight markers are indicated on the right-hand side of the figure.

Comparison of the toxicity of 100 nM amounts of the purified nicked and unnicked preparations of EtxAB revealed that they were equally active in inducing electrogenic Cl− secretion by T84 cells (Fig. 3). This is consistent with our previous finding that T84 cells contain a serine protease that can nick and activate the A-subunits of cholera toxin and E. coli enterotoxin, and it further demonstrates that nicking per se is not the rate-determining step defining the magnitude and time course of Cl− secretion.

FIG. 3. Ctx and Etx exhibit differential toxicity in T84 cells. Time course of electrogenic Cl− secretion induced by the addition of 100 nM nicked CtxAB (●), nicked EtxAB (■), or unnicked EtxAB (○) to the apical surface of T84 cell monolayers (with the data points representing the mean ± S.E., where n = 2 independent monolayers). Twelve independent experiments gave similar results.

Is the K(R)DEL Motif Responsible for Differential Toxicity?—The Lys-Asp-Glu-Leu-COOH (KDEL) motif at the C terminus of the A2-fragment of cholera toxin has been demonstrated to contribute to the efficiency of toxin action in T84 cells (22). In EtxAB, the same motif contains a conservative amino acid replacement of Arg for Lys (Fig. 4). Although the RDEL motif is known to serve as a functional ER retention signal (44, 45) and enhances EtxAB activity in T84 cells (22), the possibility remained that the differential toxicity of cholera toxin and E. coli enterotoxin might be attributable to the subtle alteration in this C-terminal motif. To investigate this, the KDEL motif of cholera toxin was replaced by an RDEL sequence, and resultant toxin, CtxA(RDEL)CtxB, was expressed and purified as described above (Fig. 5, lane 3). When the effects of 100 nM CtxA(RDEL)CtxB and wild-type CtxAB on T84 cells were compared, both toxins elicited similar anamnestic responses, with lag periods of ~30 min (Fig. 6). We therefore conclude that the different ER retention signals of cholera toxin and E. coli enterotoxin are not the cause of their differential toxicity. These findings are consistent with those of Kreitman and Pas-Gan (46), who found that KDEL or RDEL attached to the C terminus of Pseudomonas exotoxin A were equally effective in enhancing toxicity (46).

Construction of Hybrid Ctx/Etx Toxins to Determine the Basis for Differential Toxicity—To define whether the A- or B-subunits of the two toxins are responsible for their different activities in T84 cells, a set of hybrid toxins was constructed. A schematic representation of the holotoxin structure of EtxAB is shown in Fig. 4i. It illustrates that the A2-fragment can be considered as two discrete structural segments primarily responsible for interacting with the A1- and B-subunit pentamer; a long α-helix in A2 (residues 197–224) abuts the A1-fragment, while the remainder (A2 residues 225–236) winds through the pore of the toroidal B-pentamer. Because of the relatively large number of non-conservative differences in amino acids in the A2-fragments of CtxA and EtxA (Fig. 4ii),
hybrid toxins were designed with the fusion site located at the end of the long α-helix of the A2-fragment, corresponding to amino acid 225. By so doing, the interactions between the A1- and A2-fragments and between the A2-fragment and B-subunits in the hybrids would involve homologous toxin domains, which we considered would favor toxin assembly and stability. Construction of the hybrid toxins, with a fusion joint at codon 225 of the A-subunit gene, involved a series of PCR amplifications of the ctxAB and etxAB operons. The 5′-portion of the ctxA and etxA genes (to codon 226) were amplified and cloned separately as EcoRV/KpnI fragments, while the downstream 3′-portions (from codon 226 of ctxA and etxA, together with the entire ctxB and etxB gene, respectively) were cloned as KpnI/SpeI fragments. Consequently, the ligation of the 5′ and 3′ portions at the KpnI site (Fig. 1) enabled the generation of two chimeric operons, one comprising codons specifying the signal peptide plus residues 1–225 of ctxA, 226–240 of etxA, and the entire etxB gene (to yield a hybrid toxin designated CtxA (1–225)CtxA(226–240)CtxB, and the other codons specifying the signal peptide and 1–224 of etxA, 225–240 of ctxA, and the entire etxB gene (to yield a hybrid toxin designated EtxA(1–224)ctxA(226–240)ctxB). The generation of these chimeric operons resulted in the introduction of a KpnI site, spanning codons 224–226 of the A-subunit genes; this had no effect on the encoded amino acid sequence in CtxA, but changed codon 225 of ctxB to that normally found in CtxA, i.e. from an Asp to Gly, accounting for the designation of EtxA(1–224)ctxA(225–240)ctxB.

The two hybrid toxins were expressed and purified (Fig. 5, lanes 4 and 5) using conditions identical to those employed for the wild-type and RDEL mutant toxins, above, then assayed for their capacity to induce electrogenic Cl− secretion by T84 cells. The hybrid toxin CtxA(1–225)CtxA(226–240)ctxB was less potent than wild-type CtxAB at inducing Cl− secretion (Fig. 7A). Indeed the delay in the time course of Cl− secretion with the hybrid more closely resembled that of wild-type CtxAB (Fig. 7B). By contrast, the activity of the other hybrid, EtxA(1–224)ctxA(225–240)ctxB, more closely resembled that of wild-type EtxAB (Fig. 7A). These findings demonstrated that a potent electrogenic response could be elicited by toxins in which the first 224 amino acids were derived from either CtxA or EtxA. Since this region contains the entire ADP-riboylating A1-fragment (i.e. residues 1–192), we conclude that the differential toxicity of cholera toxin and E. coli enterotoxin cannot be due to differences in the enzymatic activities of the A-subunits. This conclusion was further substantiated by analysis of the intrinsic ADP-riboyltransferase activity of both the wild-type and hybrid toxins (Table I).
Differential Toxicity of Cholera Toxin and E. coli Enterotoxin

**TABLE I**

| Toxin                        | ADP-ribosyltransferase activitya | + rhARF b | - rhARF b |
|------------------------------|---------------------------------|-----------|-----------|
| Wild-type CtxAB              | 3.5 ± 0.4                       | 22.7 ± 1.0|           |
| CtxA(RDEL)CtxB               | 4.0 ± 0.4                       | 19.3 ± 0.6|           |
| CtxA(1–225)EtxA(226–240)EtxB| 2.6 ± 0.4                       | 17.7 ± 0.9|           |
| CtxA(RDEL)EtxB               | 3.3 ± 0.7                       | 25.3 ± 2.4|           |
| Wild-type EtxAB              | 6.4 ± 0.3                       | 37.3 ± 3.9|           |
| CtxA(1–225)EtxA(226–240)CtxB | 9.7 ± 0.7                       | 64.4 ± 4.6|           |

a ADP-ribosyltransferase activity determined by the method of Moss et al. (30).

b ADP-ribosyltransferase activity in the presence or absence of recombinant human ADP-ribosylation factor 6 (rhARF6).

Agmatine was used as the substrate for ADP-ribosylation, and the ADP-ribosylating activity measured with or without recombinant hARF6. Comparison of wild-type cholera toxin with the various toxin constructs containing the CtxA1-fragment, revealed that they all had similar ADP-ribosyltransferase activity. The activities of wild-type E. coli enterotoxin and the hybrid toxin containing the Etx A1-fragment were found to be approximately 1.6–2.8-fold higher than that exhibited by cholera toxin (Table I). Thus, the lower potency of E. coli enterotoxin in T84 cells cannot be attributed to a lower level of intrinsic enzymatic activity. Lee et al. (47) also noted that the activity of E. coli enterotoxin, in the agmatine assay, was slightly higher than that of cholera toxin (47), but the reasons for this remain unknown.

Taken together, the findings implicate either the C-terminal portion of the A2-fragment (from residues 226–240) or the B-subunits of the two toxins, as important determinants of the differential toxicity in vitro. Given that the B-subunits of E. coli enterotoxin are more promiscuous in receptor binding than the B-subunits of cholera toxin, one possible explanation for the lower potency of EtxAB was that differential binding to receptors might influence the efficiency of toxin delivery into T84 cells.

To address this question, a third hybrid toxin was constructed. The EcoRV-EcoRI fragment from plasmid pATA14 was substituted for the same fragment in pRC19 to generate a hybrid composed of codons specifying the signal peptide and residues 1–236 of ctxA, 237–240 of etxA, and the entire etxB gene (Fig. 1). The resultant toxin comprising the entire A-subunit of cholera toxin with a C-terminal RDEL sequence and the entire B-subunit of E. coli enterotoxin, was designated CtxA(RDEL)EtxB. Since the presence of a KDEL or RDEL sequence at the C terminus of the A-subunit had no effect on the relative potency of the toxin in T84 cells (see above), the CtxA(RDEL)EtxB hybrid allowed us to determine whether the basis of differential toxicity is due to the C-terminal portion of the A2-fragment (from residue 226) or to the B-subunit.

The A2-fragment is the determinant of differential toxicity. Purified CtxAB, CtxA(1–225)EtxA(226–240)EtxB, and CtxA(RDEL)EtxB (Fig. 5, lanes 2, 5, and 8) were tested on T84 cells at various concentrations. In all cases the CtxA(1–225)EtxA(226–240)EtxB hybrid was less effective than CtxAB and CtxA(RDEL)EtxB in triggering electrogenic Cl− secretion, even at fully saturating concentrations of 600 nM (Fig. 8). Importantly, the time course and magnitude of Cl− secretion elicited by CtxAB and CtxA(RDEL)EtxB were nearly identical (Fig. 8). Since these two toxins contain different B-subunit components,
but are equally effective in triggering Cl− secretion in T84 cells, the differential toxicity of cholera toxin and E. coli enterotoxin cannot be associated with their respective B-subunits. By contrast, the data shown in Fig. 8 demonstrate that CtxA(RDEL)EtxB and CtxA(1–225)EtxA(226–240)EtxB, which only differ in the C-terminal portion of the A-fragment, have toxicities characteristic of cholera toxin and E. coli enterotoxin, respectively. We therefore conclude that the structural basis for differential toxicity resides in the A2-fragment.

The sequences of CtxA(RDEL)EtxB and CtxA(1–225)EtxA(226–240)EtxB only differ by 4 amino acids (Fig. 4ii): Asp-229, Ile-230, Thr-232, and His-233 (in CtxA) and Glu-229, Val-230, Ile-232, and Tyr-233 (in EtxA). The residues at these positions in CtxA(RDEL)EtxB are those normally found in the A-subunit of cholera toxin, whereas those present in CtxA(1–225)EtxA(226–240)EtxB are normally found in the A-subunit of E. coli enterotoxin. We conclude that one or more of these residues influence toxin action in T84 cells. Given that the amino acid differences are in the A2-fragment that winds through the central pore of the B-pentamer (see Fig. 4i), a number of possible explanations for the difference in toxicity can be considered.

The A2-fragment from residues 225 to 240 in cholera and E. coli enterotoxin is the most structurally different region of the two toxins, as revealed by x-ray crystallography (4, 6). In E. coli enterotoxin, this segment comprises an extended chain (residues 227–231), a small α-helix (232–236), and the last four RDEL residues. The extended chain and α-helix are located within the central pore of the B-pentamer, while the exact location and structure of the RDEL sequence remain uncertain due to lack of definition in the electron density. Many of the residues in this segment are involved in intersubunit salt bridge, hydrophobic, and hydrogen bond interactions with the B-subunits. These include Glu-229, Ile-232, and Tyr-233, which interact with specific residues in the B-subunit pentamer (4). The x-ray crystal structure of cholera toxin revealed that the A2-segment from residue 227 enters the central pore of the B-pentamer as an α-helix rather than as an extended chain (6). The last four (KDEL) residues were also visible in the electron density of cholera toxin and may reflect the more compact structure of the Ctx A2- than the Etx A2-fragment. Given these structural differences, a possible explanation for differential toxicity would be that amino acid changes between cholera toxin and E. coli enterotoxin alter the stability of A/B-subunit interaction, which may in turn influence maintenance of the holotoxin structure during its transport along the endocytic pathway or potentiate liberation of the A-subunit at a stage when translocation occurs.

To test the possibility that the four amino acid differences between CtxA(RDEL)EtxB and CtxA(1–225)EtxA(226–240)EtxB resulted in a change in holotoxin stability, each hybrid was subjected to a range of in vitro conditions that might normally be expected to cause protein denaturation. The toxins were bound to Ga1-ganglioside-coated microtiter plates, treated with denaturant, washed, and probed with monoclonal antibodies specific for CtxA or EtxB as described under “Experimental Procedures.” The hybrids were found to be remarkably resistant to denaturants, maintaining their holotoxin structure in the presence of 8 M urea and at pHS as low as 4.0. These findings are not unexpected, given the exceptional stability of the cholera toxin and E. coli enterotoxin B-pentamers (11). When the hybrids were incubated in the presence of 0.5% (w/v) SDS at pH 7.2 for 30 min, the A-subunits were released from Ga1-bound B-subunits, and this was greater at pH 5.5 (Fig. 9). Of particular interest was the finding that CtxA(RDEL)EtxB (see H1 in Fig. 9) was more resistant than CtxA(1–225)EtxB (see H2 in Fig. 9) to A/B-subunit dissociation, under these conditions. Given that the toxins are likely to encounter a lower pH in endosomal compartments, and the A-subunits may well be subjected to conditions that promote unfolding during translocation, these in vitro conditions provide an important insight into holotoxin stability. Since CtxA(RDEL)EtxB is the more stable of the two toxins under these in vitro conditions, as well as being more active in inducing electrogenic Cl− secretion in T84 cells, we hypothesize that the difference in toxicity of cholera toxin and E. coli enterotoxin is due to the greater ability of cholera toxin to maintain its holotoxin structure during transit through the endocytic pathway and thus to deliver a greater proportion of internalized toxin to the site of translocation and action.

An alternative explanation for the difference in cholera toxin...
and *E. coli* enterotoxin activity in T84 cells may be that the structural differences in the A2-fragments, highlighted above, cause a subtle but significant change in the K/RDEL sequence. Since it is known that this sequence plays a role in the ability of toxin action in T84 cells, which presumable stems from its ability to engage the KDEL receptor involved in retrograde transport to the ER, it remains possible that the function of the KDEL sequence will be influenced by its position at the opening of the central pore of the B-pentamer. The absence of structural data on the RDEL sequence of *E. coli* enterotoxin does not allow a comparison to be made with that of the KDEL sequence of cholera toxin. It will clearly be important to determine whether the conformation of RDEL differs in CtxA(RDEL)EtxB and CtxA(1–225)EtxA(226–240)EtxB. Crystallization of these two hybrids is in progress.

Finally, it has been proposed that the amphipathic nature of the A2-fragment may endow it with the ability to insert into eukaryotic cell membranes, presumably the ER membrane during A-subunit translocation (24), the occurrence of which may depend on unfolding and release of the A-subunit from the B-pentamer. Thus, the nature of interactions between the A2-fragment and the B-pentamer may affect this step in the toxicity pathway.

**Concluding Remarks**—This study provides definitive evidence that the difference in activity of cholera toxin and *E. coli* heat-labile enterotoxin in T84 cells is due to structural elements in the C terminus of the A2-fragment. Preliminary X-ray crystallographic data on the CtxA(RDEL)EtxB pentamer hybrid have confirmed that the A2-tail penetrating the pore of the EtxB pentamer resembles that found in CtxAEB rather than EtxAB. This would be consistent with the view that the structural differences in the A2-fragments in CtxA(RDEL)EtxB and CtxA(1–225)EtxA(226–240)EtxB are likely to be of considerable significance in the pathology of cholera and traveler's diarrhea.

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Structural Basis for the Differential Toxicity of Cholera Toxin and Escherichia coli Heat-labile Enterotoxin: CONSTRUCTION OF HYBRID TOXINS IDENTIFIES THE A2-DOMAIN AS THE DETERMINANT OF DIFFERENTIAL TOXICITY

Chiara Rodighiero, Abu T. Aman, Martin J. Kenny, Joel Moss, Wayne I. Lencer and Timothy R. Hirst

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