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Deletion Analysis of the *Clostridium perfringens* Enterotoxin

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To further our knowledge of the structure-function relationship and mechanism of action of the *Clostridium perfringens* enterotoxin (CPE), a series of recombinant CPE (rCPE) species containing N- and C-terminal CPE deletion fragments was constructed by recombinant DNA approaches. Each rCPE species was characterized for its ability to complete the first four early steps in the action of CPE, putatively ordered as specific binding, a postbinding physical change to bound CPE, large-complex formation, and induction of alterations in small-molecule membrane permeability. These studies demonstrated that (i) at least 44 amino acids can be removed from the N terminus of CPE without loss of cytotoxicity, (ii) removal of the first 53 amino acids from the N terminus of CPE produces a fragment that appears to be noncytotoxic because it cannot undergo the post-binding physical change step in CPE action, (iii) removal of as few as five amino acids from the C terminus of CPE produces a noncytotoxic fragment lacking receptor binding activity, and (iv) a fragment lacking the first 44 N-terminal amino acids of native CPE formed twice as much large complex and was twice as cytotoxic as native CPE. From these structure-function results, it appears that the minimum-size cytotoxic CPE fragment comprises approximately residues 45 to 319 of native CPE. Results from these deletion fragment studies have also contributed to our understanding of CPE action by (i) independently supporting previous suggestions that binding, the postbinding physical change step, and large-complex formation represent important steps in CPE cytotoxicity and (ii) providing independent evidence confirming the putative sequential order of these early events in CPE action.

Clostridium perfringens enterotoxin (CPE) causes the symptoms associated with *C. perfringens* type A food poisoning (18) and has also been implicated in several other human and veterinary diseases (19). CPE is a 319-amino-acid single polypeptide with a molecular weight of 35,317 (2), whose primary sequence displays no homology to other proteins, except for some recently detected limited homology to several nonneurotoxic proteins produced by *Clostridium botulinum* (9). The functional significance, if any, of this limited homology is unclear.

Recent studies suggest that CPE cytotoxicity for mammalian cells (30) is a multistep process involving the following putative sequence of events: (i) CPE specifically binds to a 50-kDa mammalian plasma membrane protein, resulting in the formation of a "small" (~90-kDa) CPE-containing complex in plasma membranes (30); (ii) a postbinding physical change develops for the CPE sequestered in this small complex (5, 23, 25)—this step could represent either the insertion of CPE (or the entire small complex) into membranes or a conformational change in the small complex (12); (iii) an interaction occurs between the physically changed CPE small complex and a 70-kDa eukaryotic plasma membrane protein, resulting in the formation of a "large" (~160-kDa) CPE-containing complex in plasma membranes (23, 30, 31); and (iv) extensive plasma membrane permeability alterations develop for small molecules, e.g., ions and amino acids (15, 16, 19–21). These small-molecule permeability changes are apparently the primary lethal event for CPE-induced cytotoxicity, since they lead to numerous secondary enterotoxin-associated effects, such as the inhibition of macromolecular synthesis and the development of morphologic damage (11, 16, 22).

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Some progress has been made in elucidating the CPE structure-function relationship. Early studies demonstrated that limited trypsin (4) or chymotrypsin (3) treatment of CPE removes the first 25 or 37 N-terminal amino acids, respectively, from the enterotoxin and also increases the biologic activity of CPE by two- to threefold (3, 4). Another study (10), using a chemically derived CPE fragment, suggested that the C-terminal half of CPE may possess receptor-binding activity. This suggestion was confirmed when Hanna et al. (8) unambiguously demonstrated that a recombinant 19-kDa CPE fragment, corresponding to the C-terminal half of the CPE molecule (amino acids 171 to 319), lacked cytotoxic activity but possessed strong CPE-like receptor-binding activity. On further study (5), this nontoxic $rCPE_{171-319}$ fragment was shown to be specifically blocked at the second step in CPE action; i.e., it could not undergo the postbinding physical change that normally occurs to CPE small complex. Besides supporting the importance of this second step for CPE cytotoxicity, this result also indicated that some amino acids in the N-terminal half of CPE are necessary for completion of this event. Subcloning experiments (6), producing even smaller recombinant C-terminal CPE fragments, then further localized receptor-binding activity to the 30 C-terminal amino acids of CPE. Epitope-mapping studies (7) confirmed this assignment by demonstrating that monoclonal antibody (MAb) 3C9, a MAb which blocks the binding of native enterotoxin to its receptor (32), could react with this putative $CPE_{290-319}$ receptor-binding region (7).

Despite the progress described above, current understanding of CPE structure-function relationships remains rudimentary. To further define these relationships, a comprehensive series of recombinant CPE species containing N- and C-terminal CPE deletion fragments has been prepared in the current study. Characterization of these deletion fragments has unambiguously confirmed the importance of the C-terminal region of native CPE for receptor binding and also revealed that amino acids residing between residues 45 and 53 of the native

TABLE 1. Primers used for PCR amplification

rCPE species expressed[a]	Sequence of primer	Restriction sites used	Vector used	Construct name
$rCPE_{1–319}$	5′-CGCGGATCCGGCGATGTTAATTATAATATGCTTAGTAAC-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKFLt-1
$rCPE_{37–319}$	5′-CGCGGATCCTTAAGTGATGGATTATATGTAATAGATAAAGG-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKCTSt-2
$rCPE_{45–319}$	5′-CGCGGATCCTAGATAAAGGAGATGGTTGG-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Bam*HI/*Eco*RI	pTrc-His C	pJKC45t-1
$rCPE_{53–319}$ P−	5′-CATGCCATGGGGGAACCCTCAGTAGTT-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Nco*I/*Eco*RI	pSE420	pJKC53s-B
$rCPE_{69–319}$	5′-CGCGGATCCCAGGTACCTTTAGCCAATCA-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Bam*HI/*Eco*RI	pTrc-His C	pJK2Ft-4
$rCPE_{69–319}$ P−	Made by excising *cpe* insert from pJK2Ft-4 and ligating it into pSE420 (see text)	*Nco*I/*Eco*RI	pSE420	pJK2Fs-8
$rCPE_{103–319}$	5′-CGCGGATCCGGATTTGGAATAACTATAGGAGAA-3′ 5′-CCGGAATTCTATATGGAAGGAGAAATTAATGC-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKHPEt-2
$rCPE_{168–319}$	Made by digesting pJKFLt-1 with *Pst*I and *Eco*RI and then ligating *cpe* fragment back into pTrc-His (see text)	*Pst*I/*Eco*RI	pTrc-His B	pJKHPt-3
$rCPE_{1–289}$	5′-CGCGGATCCGGCGATGTTAATTATAATATGCTTAGTAAC-3′ 5′-CCGGAATTCATATATCAACATAATGATCTTTTACACCAT-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKC289t-5.2
$rCPE_{1–309}$	5′-CGCGGATCCGGCGATGTTAATTATAATATGCTTAGTAAC-3′ 5′-CCGGAATTCGGTTAATTTCCACTATATGATGAATTAGC-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKTyr310t-1
$rCPE_{1–314}$	5′-CGCGGATCCGGCGATGTTAATTATAATATGCTTAGTAAC-3′ 5′-CCGGAATTCGAAATTATATTGAATAAGGGTAATTTCCAC-3′	*Bam*HI/*Eco*RI	pTrc-His A	pJKC314t-1

[a] Unless otherwise noted (by P−), all recombinant CPE species are expressed as fusion proteins from pTrc-His.

enterotoxin are necessary for completion of the putative second step (the postbinding physical change to CPE in small complex) in CPE action. Furthermore, results with these CPE deletion fragments also offer important independent support for two conclusions about CPE cytotoxicity that were tentatively drawn from low-temperature analyses of native enterotoxin action: (i) large-complex formation occurs after the development of the postbinding physical change to CPE (23), and (ii) large-complex formation represents an important step in the development of CPE-induced membrane permeability alterations (23).

MATERIALS AND METHODS

Materials. CPE was prepared and purified, and its biologic activity was assayed as previously described (27). Purified CPE was radioiodinated to a specific activity of 2 to 4 mCi/mg of CPE, also as described previously (24). Rabbit intestinal brush border membranes (BBMs) were prepared from the small intestines of female New Zealand White rabbits by the method of Sigrist et al. (29). All restriction enzymes and other recombinant DNA reagents were purchased from Boehringer Mannheim (Indianapolis, Ind.), unless otherwise noted.

Cloning of *cpe* gene fragments into expression vectors. Most *cpe* fragments for expressing the recombinant CPE species used in this study were prepared by PCR amplification, as previously described (1), of specific portions of the cloned *cpe* gene (2) with the appropriate primers (Table 1). PCR products were then double digested for 16 h at the appropriate temperature with the restriction enzymes (20 U/μg of DNA) indicated in Table 1. Following gel purification, the double-digested PCR products were ligated (28), in frame, into either pTrc-His A, pTrc-His B, or pTrc-His C or pSE420 (Invitrogen, San Diego, Calif.) and competent *Escherichia coli* DH5α cells were transformed (28) by these ligation reactions. The resulting transformants were selected by overnight growth on Luria agar (28) containing ampicillin (100 μg/ml). Selected bacterial colonies on these plates were then electrophoretically screened for the presence of the expected *cpe* DNA insert by using plasmid DNA samples that had been extracted from these cells and treated with the same restriction enzymes used to construct the transformed plasmid. Clones possessing DNA inserts of the correct size were then screened for expression of the expected CPE product by Western immunoblotting (see below). DNA sequencing of each PCR-amplified *cpe* fragment confirmed that no mutations had been introduced during PCR amplification (data not shown).

Two *cpe* constructs, pJKHPt-3 and pJK2Fs-8, were prepared by restriction digestion of the *cpe* gene instead of by PCR amplification. The pJKHPt-3 construct (Table 1) was made by double digesting the pJKFLt-1 construct (Table 1) with *Pst*I and *Eco*RI and then ligating the resultant *cpe* gene fragment into the similarly double-digested pTrc-His B plasmid. The pJK2Fs-8 construct was made by digesting the pJK2Ft-4 construct (Table 1) with *Bam*HI and then using mung bean nuclease (New England BioLabs, Beverly, Mass.) to blunt the overlapping ends of the resultant plasmid (28). The *cpe* insert of pJK2Ft-4 was then excised by *Eco*RI digestion of the linearized plasmid followed by gel purification of the *cpe* insert. pSE420 was digested with *Nco*I, and the resulting overhang was blunted by filling in with Klenow (28). This vector was then digested with *Eco*RI to allow the *cpe* fragment excised from pJK2Ft-4 to be ligated, in frame, into the blunted *Nco*I site of the pSE420 expression vector. After their construction, both pJK2Fs-8 and pJKHPt-3 were transformed into competent *E. coli* DH5α.

Expression of rCPE species from the various recombinant plasmids. To express full-length recombinant CPE ($rCPE_{1–319}$) or rCPE species containing CPE deletion fragments, *E. coli* DH5α transformants were grown at 37°C in SOB medium (28) containing 300 μg of ampicillin per ml and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. At 4 h after induction, the cultures were harvested and the bacterial pellets were washed and resuspended in either Tris-EDTA-saline (12.5 mM Tris-HCl, 6.25 mM EDTA, 12.5 mM NaCl [pH 8]) or Tris-saline (50 mM Tris-HCl, 100 mM NaCl [pH 8]), depending on later usage (see below).

Crude *E. coli* lysates containing the various expressed rCPE species were prepared from bacteria frozen in 2 to 5 ml of Tris-EDTA-saline. These bacteria were sonicated to >95% lysis on ice, and the insoluble material was pelleted by centrifugation. The remaining soluble lysate material was dialyzed against phosphate-buffered saline (PBS) (23).

Talon Metal Affinity Resin enrichment and EnterokinaseMax cleavage of rCPE species. Recombinant *E. coli* cultures frozen in 10 to 20 ml of Tris-saline were used for Talon Metal Affinity Resin (Clontech, Palo Alto, Calif.) enrichment of the $(His)_6$-tagged rCPE species expressed from pTrc-His-based constructs (Table 1), as specified by the manufacturer. Chromatography fractions

identified as containing rCPE species by Western immunoblotting were pooled and dialyzed overnight at 4°C against 50 mM Tris-HCl (pH 8) to remove imidazole and NaCl.

A portion of each dialyzed, affinity-enriched rCPE preparation was incubated with EnterokinaseMax (Invitrogen), as specified by the manufacturer, to remove the $(His)_6$-containing fusion peptide added by the pTrc-His expression vector to the N terminus of these rCPE species. The remainder of each preparation of affinity-enriched rCPE species was similarly incubated in EnterokinaseMax buffer alone (i.e., without enterokinase) as a control.

Quantitation of rCPE concentrations in *E. coli* lysates or affinity-enriched preparations. rCPE species (containing either $rCPE_{1-319}$ or an N-terminal CPE deletion fragment) in crude *E. coli* lysates, or affinity-enriched preparations of these rCPE fusion proteins (as available), were analyzed by Western immunoblotting, performed as previously described (1, 2, 13). The primary antibody was either immunoglobulin G (IgG) purified from rabbit polyclonal anti-CPE serum (RPC-IgG), prepared as described previously (17), for qualitative analysis or MAb 3C9 (32) for quantitative analysis of these rCPE species (see Results); when MAb 3C9 was used, blots were subsequently incubated with a rabbit anti-mouse IgG (Sigma, St. Louis, Mo.) to facilitate later detection with ^{125}I-protein A. The amount of these rCPE species present in a particular preparation was densitometrically quantitated by comparing the MAb 3C9 immunoreactivity of the preparation with the MAb 3C9 immunoreactivity of a dilution series of purified native CPE (2). Values obtained by these quantitative MAb3C9 Western immunoblots were adjusted for differences in the molecular weight of each rCPE species to calculate the molarity of the rCPE species present in each preparation. Quantitation was performed twice on three different sample volumes for each lysate preparation or affinity-enriched sample. Preliminary control experiments (data not shown) established that equivalent immunoreactivities were obtained whether the dilution series of purified native CPE was dissolved in PBS or negative-control *E. coli* lysate (i.e., lysates from DH5α transformed with pTrc-His or pSE420 vectors without a *cpe* insert).

In some experiments, Western immunoblots were probed, as specified by the manufacturer, with a Ni-nitrilotriacetic acid alkaline phosphatase conjugate (Qiagen, Chatsworth, Calif.) specific for detecting the $(His)_6$ metal-binding motif of the fusion peptide of rCPE species expressed from pTrc-His expression vectors.

Because SDS treatment of rCPE species containing C-terminal deletion fragments significantly reduced the immunoreactivity of these rCPE species with polyclonal antibodies (see Results), the concentrations of rCPE species containing C-terminal CPE deletion fragments, whether present in *E. coli* lysate or affinity-enriched preparations, was estimated by using quantitative native immunodot blots instead of Western immunoblots. Briefly, different volumes (1 to 5 μl) of *E. coli* lysate, or affinity-enriched material containing these rCPE species, were adjusted to a total volume of 5 μl with sterile double-deionized H_2O and these samples were dotted onto Immobilon-NC (Millipore, Bedford, Mass.) and allowed to air dry. The immunoreactivity in each sample was developed as described above for Western immunoblots by using RPC-IgG (2, 13), and this immunoreactivity was then compared with the immunoreactivity of a dilution series of purified native CPE to quantitate the amount of rCPE species present in a sample. Assays were performed twice in triplicate for each sample. Preliminary control experiments (data not shown) established that equivalent immunoreactivity was obtained whether the dilution series of purified native CPE was dissolved in PBS or negative-control *E. coli* lysate.

Binding inhibition by rCPE species. The receptor-binding ability of each recombinant CPE species was determined by evaluating its ability to competitively inhibit ^{125}I-CPE binding to BBMs. This was accomplished by preincubating BBMs (100 μg of protein) in a total reaction volume of 200 μl for 5 or 20 min at 22°C with one of the following: (i) increasing concentrations (0.001 to 5,000 nM) of native CPE, dissolved in the presence or absence of increasing volumes of negative-control *E. coli* lysate (0.1 to 200 μl per sample); (ii) an *E. coli* lysate containing a rCPE species (at a 0.001 to 5,000 nM final concentration of each rCPE species, which corresponded to 0.01 to 200 μl of lysate); (iii) negative-control *E. coli* lysates alone (0.1 to 200 μl per sample); (iv) an affinity-enriched preparation of each $(His)_6$-tagged rCPE species (concentrations as specified below in the text) prepared in this study; or (v) EnterokinaseMax-treated, affinity-enriched rCPE species preparations (concentrations as specified below in the text). This preincubation step was followed by the addition of 0.5 μg of ^{125}I-CPE and sample processing as previously described (6). The competitive binding activity of each rCPE species was expressed as the percentage of control ^{125}I-CPE binding remaining in the presence of specified concentrations of each unlabeled rCPE species competitor, which was calculated as the ratio between the counts per minute of ^{125}I-CPE bound to BBMs in the presence of each concentration of rCPE species tested and the counts per minute of ^{125}I-CPE bound to BBMs preincubated in PBS alone (which was considered control binding).

^{125}I-CPE binding inhibition due to the *E. coli* lysate background itself, if any, was corrected for each sample pretreated with a rCPE species-containing *E. coli* lysate by subtracting out any ^{125}I-CPE binding-inhibitory effects detected when an identical BBM sample was preincubated with an equivalent volume of negative-control (i.e., no rCPE species present) *E. coli* lysate sample.

Two control experiments were conducted to confirm that all corrected reductions in ^{125}I-CPE binding attributed to rCPE species-containing *E. coli* lysates (as determined above) were, in fact, due to the competitive binding activity of the rCPE species present in these lysates. First, the ability of MAb 3C9 to neutralize the binding inhibition activity attributed to the various rCPE species in *E. coli* lysates was evaluated, as previously described (6). Second, to further confirm conclusions being drawn about the binding abilities of each rCPE species with *E. coli* lysate samples, similar preincubation experiments were repeated, in the absence of any background *E. coli* lysate material, with 500 nM (final concentrations) affinity-enriched rCPE preparations (as available) that had (or had not) been subjected to enterokinase cleavage.

Detection of the postbinding physical change in the rCPE species. Protection of each rCPE species from pronase-induced release from BBMs was used as a well-established (23, 25) indicator of the ability of each rCPE species to perform the postbinding physical-change step of CPE action. *E. coli* lysates containing one of the specified rCPE species (or added native CPE) or affinity-enriched rCPE (both enterokinase cut and uncut) were incubated at final CPE concentrations of 100 nM with 100 μg of BBMs in a 200-μl total volume of PBS for 5 or 20 min at 22°C. These BBMs were pelleted by microcentrifugation and then washed twice with 200 μl of PBS to remove any unbound CPE species. One set of these washed BBMs was then incubated for 5 min at 22°C with 25 μg of pronase (Boehringer Mannheim) in 200 μl of PBS, while a second set of these washed BBMs was incubated for 5 min without pronase. After this incubation, both sets of samples were pelleted by microcentrifugation and washed twice with 200 μl of PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Each washed BBM pellet was then extracted for 20 min at 22°C with 50 μl of PBS containing 1% Triton X-100 (Sigma), and 2 mM PMSF. A 40-μl sample of the extracted BBMs was dotted on nitrocellulose in eight repetitions of 5-μl dots (each), and the dots were allowed to air dry between repetitions. As controls, (i) the same concentration of either an rCPE species (or added native CPE) in *E. coli* lysate or an affinity-enriched rCPE species (either enterokinase cut or uncut) as used above was directly treated with 25 μg of pronase, followed by the addition of Triton X-100 (to a 1% final concentration) plus 2 mM PMSF, before being dotted on nitrocellulose and (ii) similar concentrations, as above, of a rCPE species (or added native CPE) in *E. coli* lysate were dissolved in PBS containing 1% Triton X-100 and directly dotted onto nitrocellulose without any pronase treatment. After dotting, the nitrocellulose sheet containing all the samples was processed as described for Western immunoblots, with RPC-IgG and ^{125}I-protein A.

Large-complex formation by rCPE species. The ability of each rCPE species to form CPE large complex (31) was examined by Western immunoblotting of CPE large-complex gels (12). Briefly, *E. coli* lysates containing each rCPE species (or added native CPE) or the affinity-enriched rCPE species [with or without the $(His)_6$-containing peptide present, as appropriate] were incubated, at 25, 50, or 100 nM final concentrations of each CPE species, with 100 μg of BBMs in a total volume of 200 μl of PBS for 5 or 20 min at 22°C and then analyzed by Western immunoblotting of large-complex gels. Densitometry for quantitation of large-complex formation by each rCPE species was determined as described above.

Assay for Vero cell cytotoxicity. The release of ^{86}Rb from radiolabeled Vero cells was used to measure the CPE-like cytotoxicity of the various rCPE species. These assays were conducted as previously described (16, 23, 24), with samples including (i) increasing concentrations (0.2 to 200 nM, final concentrations) of native CPE added to negative-control *E. coli* lysate or one of our recombinant CPE species in *E. coli* lysate, or (ii) 0.2 to 200 nM (final concentrations) each affinity-enriched rCPE species [with or without the $(His)_6$-containing peptide present] for 15 min. In one time-course experiment, samples containing a single final concentration (7 nM) of CPE or of affinity-enriched $rCPE_{1-319}$, $rCPE_{37-319}$, or $rCPE_{45-319}$ species [with or without the $(His)_6$-containing peptide still present] were incubated with Vero cells for 1 to 20 min, as specified. The percent maximal release was calculated as $100 \times$ (release due to sample − spontaneous release)/(maximal release − spontaneous release).

Maximal release was 3×10^3 to 4×10^3 cpm per culture, and spontaneous release after 15 min was 0.5×10^3 to 1×10^3 cpm per culture; addition of equivalent volumes of negative-control *E. coli* lysate to wells did not cause any ^{86}Rb release above spontaneous release values (data not shown). To further ensure that all observed ^{86}Rb release above background observed with some rCPE species was, in fact, being mediated by the rCPE species present in that sample, 20 nM (final concentration) each cytotoxic rCPE species was preincubated for 20 min at 37°C in the presence or absence of a 50-fold excess of MAb 3C9 or MAb 10G6 (32) (a nonneutralizing anti-CPE MAb) before being used in this assay.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (14), with bovine serum albumin as the protein standard.

RESULTS

Expression of rCPE species in *E. coli* lysates. A series of recombinant *E. coli* DH5α transformants, each carrying a plasmid encoding an rCPE species containing either $rCPE_{1-319}$ or an N- or C-terminal CPE deletion fragment, was prepared (Table 1). Western immunoblot analysis (Fig. 1 contains representative results) demonstrated that each transformant ex-

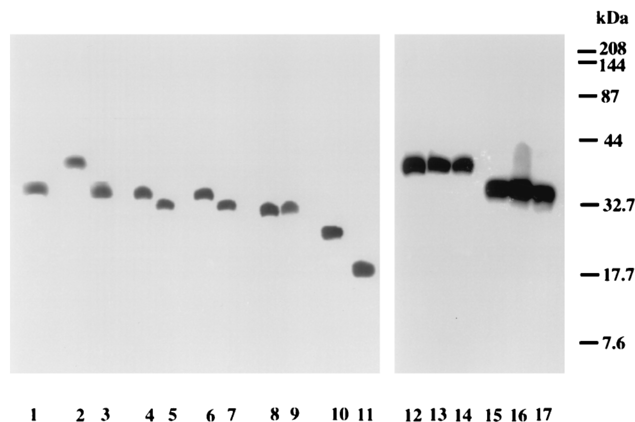

FIG. 1. (Left) Western immunoblot analysis of affinity-enriched preparations of rCPE species containing $rCPE_{1-319}$ and each of the N-terminal CPE deletion fragments expressed from pTrc-His, probed with RPC-IgG. Samples shown include native CPE (0.2 μg [lane 1]), $rCPE_{1-319}$ (2 μl [lanes 2 and 3]), $rCPE_{37-319}$ (1 μl [lanes 4 and 5]), $rCPE_{45-319}$ (1 μl [lanes 6 and 7]), $rCPE_{103-319}$ (15 μl [lanes 8 and 9]), and $rCPE_{168-319}$ (0.5 μl [lanes 10 and 11]). (Right) Western immunoblot of affinity-enriched preparations of rCPE species containing each of the C-terminal CPE deletion fragments, probed with RPC-IgG. Samples shown include $rCPE_{1-314}$ (40 μl [lanes 12 and 15]), $rCPE_{1-309}$ (40 μl [lanes 13 and 16]), and $rCPE_{1-289}$ (40 μl [lanes 14 and 17]). Samples shown in lanes 2, 4, 6, 8, 10, 12, 13, and 14 are affinity-enriched preparations of rCPE fusion proteins, while those shown in lanes 3, 5, 7, 9, 11, 15, 16, and 17 are affinity-enriched preparations of each fusion protein after treatment with EnterokinaseMax.

pressed a single polypeptide reactive with RPC-IgG; this immunoreactive material always matched the expected size of the rCPE species encoded by each transformant. No immunoreactive material was detected on Western immunoblots of negative-control lysates prepared from *E. coli* DH5α transformed with either the control pTrc-His or control pSE420 vectors alone (data not shown).

When Western immunoblot experiments were repeated with anti-CPE MAb 3C9, lysates containing either (i) full-length $rCPE_{1-319}$ fusion protein or (ii) N-terminal CPE deletion fragment fusion proteins expressed from pTrc-His or pSE420 displayed similar immunoreactivity to that shown with RPC-IgG in Fig. 1 (data not shown). However, *E. coli* lysates containing any of our C-terminal CPE deletion fragment fusion proteins were not reactive with MAb 3C9 on Western immunoblots (data not shown), consistent with previous studies (7) suggesting the native CPE molecule contains a single linear MAb 3C9 epitope located at its extreme C terminus. Therefore, MAb 3C9 Western immunoblots were used throughout our studies to quantitate the amounts of recombinant CPE species containing N-terminal CPE deletion fragments that were being added to experimental samples.

Western immunoblot studies also showed that, on a molar protein basis, rCPE species containing C-terminal CPE deletion fragment fusion proteins were ~40-fold less immunoreactive with RPC-IgG than were rCPE species containing N-terminal CPE deletion fragments (Fig. 1). Removal of the $(His)_6$-containing peptide from any fusion protein containing a C-terminal CPE deletion fragment (see below) did not noticeably improve the RPC-IgG immunoreactivity of these fragments on Western immunoblots (Fig. 1). However, it was observed that all rCPE species containing C-terminal CPE deletion fragments (whether present in lysates or in affinity-enriched preparations, with or without enterokinase cleavage [see below]) were strongly reactive with RPC-IgG (but not with MAb3C9) on native immunodot blots (data not shown). In contrast to the ~40-fold differences in Western immunoblot immunoreactivity between rCPE species containing C-terminal versus N-terminal CPE deletion fragments described above, rCPE species containing C-terminal CPE deletion fragments exhibited, on a molar protein basis, only one- to twofold less RPC-IgG reactivity on native immunodot blots than did native CPE, $rCPE_{1-319}$, or rCPE species containing N-terminal CPE deletion fragments. Therefore, RPC-IgG native immunodot-blots comparing the immunoreactivity of preparations of each rCPE species containing a C-terminal CPE deletion fragment against the RPC-IgG immunoreactivity of known amounts of native CPE were used in our studies to quantify the approximate concentrations of these rCPE species being added in each experiment described below, with the understanding that this approach is conservative and actually understates the amount of rCPE species containing the C-terminal deletion fragment that was being added to an experiment by ~50%. It was also observed (data not shown) that MAb 10G6 (which recognizes a conformational epitope involving N-terminal sequences of CPE [7, 32]) reacts equally well on native immunodot blots with both rCPE species containing C-terminal CPE deletion fragments and native CPE. However, this immunoreactivity was considered too weak for reliable quantitation of rCPE species concentrations by densitometric analysis.

Affinity enrichment of rCPE species encoded by pTrc-His-based constructs. Since most rCPE species expressed from our plasmid constructs (Table 1) were fusion proteins containing a 32-amino-acid $(His)_6$-containing N-terminal peptide encoded by pTrc-His, it was possible to use metal affinity chromatography to enrich these rCPE species from *E. coli* lysates. Western immunoblot analysis with RPC-IgG confirmed that each such affinity-enriched preparation contained a single immunoreactive species corresponding to the expected size of the vector-

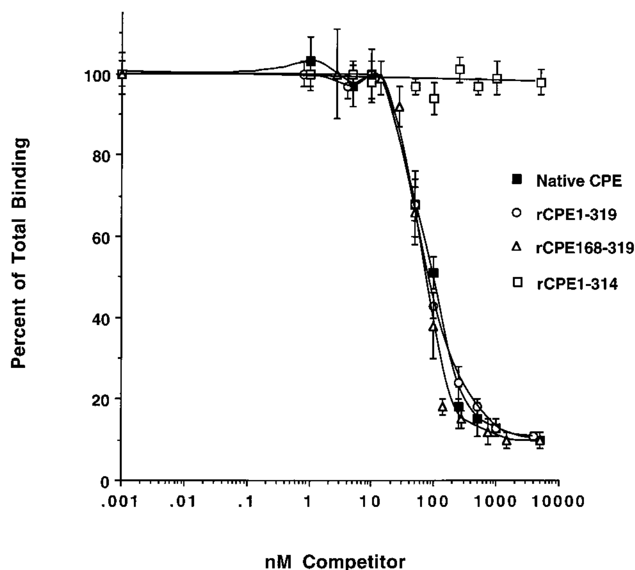

FIG. 2. Competitive inhibition of ^{125}I-CPE binding to BBMs by representative rCPE species. Increasing final concentrations (0.001 to 5,000 nM) of competitor, i.e., *E. coli* lysates containing either $rCPE_{1-319}$, $rCPE_{168-319}$, or $rCPE_{1-314}$ fusion proteins, or added native CPE were preincubated with BBMs after ^{125}I-CPE was added. The percentage of control binding in the presence of each competitor was determined as described in Materials and Methods. (All other rCPE species containing N-terminal CPE deletion fragments also displayed competitive binding properties very similar to $rCPE_{1-319}$, while no rCPE species containing C-terminal CPE deletion fragments displayed any competitive binding activity [data not shown].) The results are mean values obtained from triplicate samples in three independent experiments. Error bars shown represent standard deviations; points without error bars had standard deviations too small to depict.

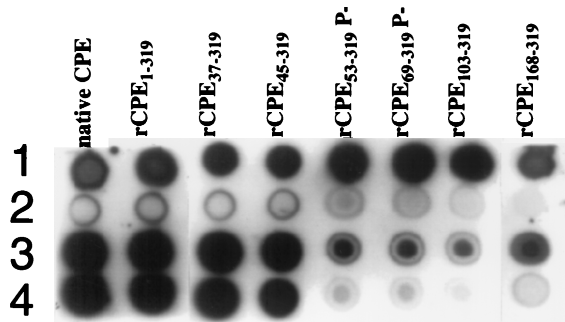

FIG. 3. Postbinding physical change by rCPE species containing N-terminal CPE deletion fragments. Binding-capable rCPE species containing N-terminal CPE deletion fragments (as noted on the figure) were tested for their ability to complete the second step in CPE action, i.e., to become resistant to pronase-induced release from BBMs. Samples dotted on nitrocellulose are as follows: (row 1) *E. coli* lysates with 100 nM final concentrations of either added native CPE or an rCPE species (either $rCPE_{1-319}$, one of the various N-terminal CPE deletion fusion proteins, $rCPE_{53-319}$ P−, or $rCPE_{69-319}$ P−) dissolved in PBS containing 1% Triton X-100 and 2 mM PMSF; (row 2) same samples as in row 1, except for treatment with 25 μg of pronase for 5 min prior to the addition of 1% Triton X-100 and 2 mM PMSF; (row 3) *E. coli* lysates with 100 nM final concentrations of the CPE species described in row 1 incubated with BBMs for 20 min prior to extraction with PBS containing 1% Triton X-100 and 2 mM PMSF; and (row 4) same samples as shown in row 3, except that the CPE-treated BBMs had been incubated with 25 μg of pronase prior to extraction with the PBS containing 1% Triton X-100 and 2 mM PMSF. The nitrocellulose sheet containing rows 1 to 4 was processed with RPC-IgG followed by ^{125}I-protein A, as described in Materials and Methods.

encoded fusion peptide (M_r 3,900) plus either an N-terminal or C-terminal CPE deletion fragment (Fig. 1). Coomassie blue staining of affinity-enriched samples subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that each preparation contained only a single stained band (data not shown), which always comigrated with the immunoreactive rCPE species present in these samples, indicating that each affinity-enriched preparation contained a virtually pure rCPE species.

To evaluate, in experiments described below, whether attributed functions (or lack of functions) of each rCPE species were being affected by the presence of the vector-encoded N-terminal peptide present on our pTrc-His-encoded rCPE species, enterokinase was used to selectively remove the $(His)_6$-containing N-terminal peptide from most affinity-enriched rCPE fusion proteins [it was also observed (data not shown) that enterokinase did not remove the $(His)_6$-containing peptide from rCPE fusion proteins if these fusion proteins were still present in crude *E. coli* lysates]. As shown in Fig. 1, enterokinase treatment reduced the size of all rCPE fusion proteins, except for the $rCPE_{103-319}$ fusion protein (which presumably has a conformational perturbation rendering it enterokinase resistant), by ~4kDa, a size reduction corresponding to the expected removal of the vector-encoded peptide from these fusion proteins. The conclusion that enterokinase treatment was specifically removing the $(His)_6$-containing N-terminal peptide from these fusion proteins is further supported by results (data not shown) demonstrating that enterokinase treatment caused fusion proteins to lose reactivity with a Ni-nitrilotriacetic acid conjugate probe specific for $(His)_6$-containing peptides. Furthermore, it was also determined that enterokinase treatment of native CPE did not affect the size of the enterotoxin or its ability to perform any CPE activity described below (data not shown).

It was found during our studies that the amounts of soluble $rCPE_{69-319}$ or $rCPE_{53-319}$ fusion proteins present in *E. coli* lysates were too small to allow metal affinity chromatography of these rCPE species, thus also preventing the use of enterokinase cleavage (see above) to remove the $(His)_6$-containing peptide from these $rCPE_{69-319}$ and $rCPE_{53-319}$ fusion proteins. Therefore, to obtain usable quantities of $rCPE_{53-319}$ and $rCPE_{69-319}$ fragments free from substantial vector-encoded amino acids for later use in functional activity experiments, it was necessary to express these two rCPE species (named $rCPE_{53-319}$ P− and $rCPE_{69-319}$ P−) from pSE420, which does not add a vector-encoded peptide to expressed protein products.

Functional characterization of rCPE species. Because expression of $rCPE_{53-319}$ P− and $rCPE_{69-319}$ P− without a $(His)_6$-containing peptide precluded metal affinity chromatography enrichment of these rCPE species from *E. coli* lysates, all functional characterization experiments were initially conducted with rCPE species present in *E. coli* lysates and then repeated, with available rCPE species, using affinity-enriched material.

(i) Characterization of binding properties of rCPE species. When BBMs were preincubated for 5 min (data not shown) or 20 min (Fig. 2 shows representational data) with *E. coli* lysates containing the specified final molar concentrations of added native CPE, $rCPE_{1-319}$ fusion protein, or any rCPE species containing a N-terminal CPE deletion fragment prior to the addition of ^{125}I-CPE, ^{125}I-CPE binding to these pretreated BBMs was inhibited to the same extent, on a molar basis, by each of these CPE species. The representative binding inhibition data shown in Fig. 2 is not explainable by the *E. coli* lysate background itself, since nonspecific lysate effects have already been corrected for. Note that (i) these background lysate effects on ^{125}I-CPE binding were always $<33\%$ of the total ^{125}I-CPE-binding inhibition for any sample containing added native CPE or an rCPE species, even with the maximal volume of lysate (200μl) used in these experiments, and (ii) the total protein concentrations in all lysate samples, including negative-control lysates, were determined to be approximately equal.

However, to demonstrate conclusively that all rCPE species containing N-terminal CPE deletion fragments possess CPE-like competitive binding activity, additional control experiments were performed to confirm that the active lysate agent mediating the competitive binding effects shown in Fig. 2 were, in fact, the rCPE species present in the lysate sample. In the first control experiment (data not shown), it was observed that the presence of MAb 3C9 (which blocks the binding of native CPE to its receptor [7, 32]) during the preincubation of BBMs with lysates containing an N-terminal CPE deletion fragment completely abrogated the ability of these lysates to cause a subsequent inhibition of ^{125}I-CPE binding to these pretreated BBMs. In a second control experiment, conducted in the absence of any background lysate material, it was observed that BBMs preincubated with 500 nM (final concentrations) affinity-enriched rCPE species containing any of our N-terminal CPE deletion fragments [either with or without the $(His)_6$-containing peptide present] were also unable to subsequently bind any ^{125}I-CPE.

However, when BBMs were preincubated with even very high (5,000 nM) final concentrations of any of our rCPE species containing a C-terminal CPE deletion fragment, whether present in lysates (Fig. 2 contains representational data) or in affinity-enriched preparations [with or without the $(His)_6$-containing peptide still present (data not shown)], no inhibition of ^{125}I-CPE binding, beyond the effects due to the lysate background itself (if present), was detected.

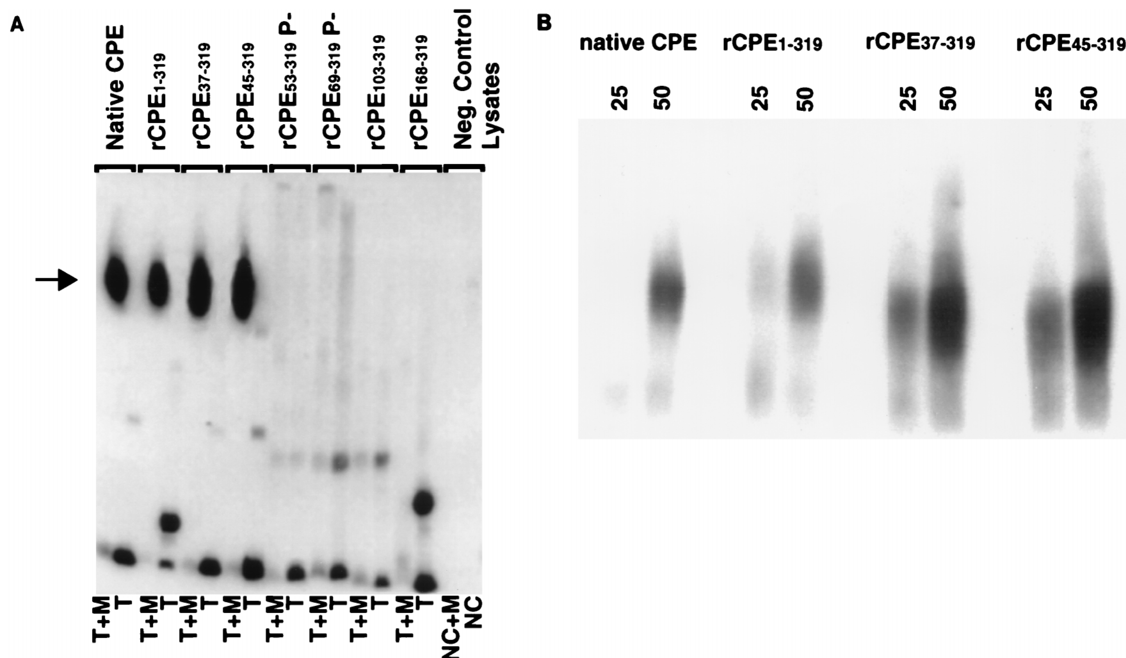

FIG. 4. Large-complex formation by various rCPE species containing the N-terminal CPE deletion fragments. (A) A survey of the ability of binding-capable rCPE species to form the CPE large complex. *E. coli* lysates with 100 nM final concentrations of either added native CPE or rCPE species (including the various N-terminal CPE deletion fragment fusion proteins, $rCPE_{53-319}$ P−, or $rCPE_{69-319}$ P−) were incubated with BBMs for 20 min and extracted with SDS-sample buffer. Large complex was detected by Western immunoblotting as previously described (12). Lanes designated T+M contain specified CPE species incubated with BBMs as described above, while lanes designated T contain the specified CPE species alone. Lane NC is a sample of negative-control *E. coli* lysate. The arrow on the left denotes where large complex formed by native CPE dissolved in PBS migrates on this gel system. Higher-molecular-weight species running below the large complex but above the bottom of the gel (present in some lanes) are SDS-induced aggregates of the various CPE species, as previously described (24). (B) Comparison of large-complex formation by CPE species capable of forming the large complex. Native CPE or affinity-enriched recombinant CPE species ($rCPE_{1-319}$, $rCPE_{37-319}$, or $rCPE_{45-319}$ fusion proteins, as noted in the figure) were incubated, at 25 or 50nM final concentrations (as noted in the figure), with BBMs for 5 min. Each sample was extracted with SDS-sample buffer and electrophoresed as described above. Densitometric analysis of the amount of large complex formed by each CPE species was determined as described in Materials and Methods.

(ii) Characterization of the ability of rCPE species to complete the postbinding physical change (step 2) in CPE action. Previous studies (12, 23, 25) have established that when membrane-bound native CPE undergoes a postbinding physical change (the putative second step in CPE action), this bound toxin becomes highly resistant to pronase-induced release from membranes. Therefore, the susceptibility of our binding-capable rCPE species (i.e., species containing an N-terminal CPE deletion fragment) to pronase-induced release from membranes was used to assess whether each of these rCPE species could complete the second step in CPE action (Fig. 3).

Samples (row 1) of native CPE or *E. coli* lysates containing either $rCPE_{1-319}$ fusion protein or an rCPE species containing a N-terminal CPE deletion fragment were able to react strongly with RPC-IgG when blotted on nitrocellulose, despite the presence of 1% Triton X-100 in these samples. Treatment of these same samples with pronase before blotting resulted in the virtually complete loss of immunoreactivity on nitrocellulose blots (row 2), confirming that the pronase preparation used in the experiments in Fig. 3 was active. When BBMs were incubated with equivalent molar amounts of these samples and then extracted with 1% Triton X-100, each of these extracted BBM samples was reactive with RPC-IgG (row 3). The immunoreactivity shown in row 3 was specifically dependent on pretreatment of BBMs with these rCPE species, since no immunoreactivity was observed when using either control BBMs or BBMs treated with negative-control *E. coli* lysates (data not shown). Time course experiments (data not shown) suggest that the somewhat lower immunoreactivity visible for BBM samples treated with *E. coli* lysates containing $rCPE_{53-319}$ P−, $rCPE_{69-319}$ P−, or $rCPE_{103-319}$ in row 3 of Fig. 3 may be due, at least in part, to greater postbinding dissociation of these rCPE species from BBMs in this assay. When identically prepared BBM samples were treated with pronase, washed, and then extracted with 1% Triton X-100, only the samples containing BBMs preincubated with lysates containing native CPE, $rCPE_{1-319}$, $rCPE_{37-319}$, or $rCPE_{45-319}$ fusion proteins still showed immunoreactivity with RPC-IgG (row 4); i.e., only these CPE species were resistant to protease-induced release from BBMs. Supporting this finding, similar results (data not shown) were observed when this experiment was repeated with affinity-enriched rCPE species [either with or without the $(His)_6$-containing peptide present], as available. Further, similar results (data not shown) were also observed if the experiments shown in Fig. 3 were repeated with shorter (5-min instead of 20-min) incubations of BBMs with these rCPE species, whether these rCPE species were present in lysates or as affinity-enriched preparations.

When these protease sensitivity experiments were repeated with rCPE species containing our three C-terminal CPE deletion fragments (data not shown), all three rCPE species (whether in lysates or affinity-enriched preparations) were observed to be immunoreactive on these dot blots and sensitive to pronase treatment prior to blotting. However, no RPC-IgG immunoreactivity was detected for BBM samples preincubated with any of the rCPE species containing a C-terminal CPE deletion fragment, confirming our findings that these three rCPE species lack binding activity.

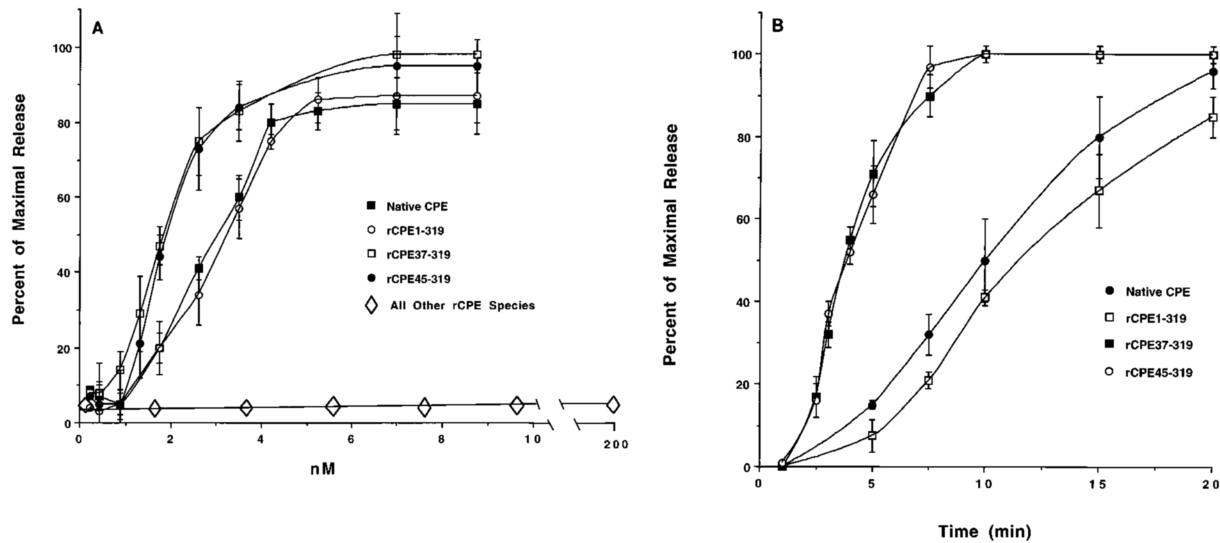

FIG. 5. Vero cell cytotoxicity. (A) The cytotoxicity of the various rCPE species for Vero cells was determined by a previously described (16, 23, 24) ^{86}Rb release assay. *E. coli* lysates containing added native CPE or a recombinant CPE species (either rCPE$_{1-319}$ fusion protein, an N-terminal or C-terminal deletion fragment fusion protein, rCPE$_{53-319}$ P−, or rCPE$_{69-319}$ P− [final concentrations, 0.2 to 200 nm]) were incubated for 15 min with ^{86}Rb-labeled Vero cells. The percentage of maximal release of the ^{86}Rb label was determined for each final concentration as described in Materials and Methods. (B) Kinetics of ^{86}Rb release for the various cytotoxic rCPE species. Vero cells labeled with ^{86}Rb were incubated with 7 nM final concentrations of native CPE or affinity-enriched rCPE$_{1-319}$, rCPE$_{37-319}$, or rCPE$_{45-319}$ fusion proteins for increasing times (1 to 20 min). The percentage of maximal release was determined as described in Materials and Methods. The results shown are mean values obtained from triplicate samples in three independent experiments. Error bars shown represent standard deviations; points without error bars had standard deviations too small to depict.

(iii) Ability of rCPE species to form the large complex. To determine whether any rCPE species prepared in this study can form the large complex (the third step in CPE action), Western immunoblot analysis of "large-complex gels" was conducted, as described previously (12). When BBMs were incubated for 20 min with equivalent final molar concentrations of the specified rCPE species present in *E. coli* lysates, the rCPE$_{1-319}$, rCPE$_{37-319}$, and rCPE$_{45-319}$ fusion proteins all formed a larger-molecular-weight species comigrating on Western immunoblots with the large complex formed by native CPE added to negative-control *E. coli* lysates (Fig. 4A). However, lysates containing fusion proteins of smaller N-terminal CPE deletion fragments did not induce the formation of the large complex (Fig. 4A). Identical results were observed (data not shown) when this experiment was repeated with affinity-enriched rCPE species [whether with or without the presence of the (His)$_6$-containing peptide], as available. No rCPE species containing a C-terminal deletion fragment, whether present in *E. coli* lysates or in affinity-enriched preparations [with or without the (His)$_6$-containing peptide], induced the formation of the CPE large complex (data not shown).

In the results in Fig. 4A, rCPE$_{37-319}$ and rCPE$_{45-319}$ fusion proteins appear to form more large complex, on a molar basis, than does either native CPE or the rCPE$_{1-319}$ fusion protein. This effect became particularly noticeable when 25 or 50 nM (final concentrations) purified native CPE or affinity-enriched rCPE$_{1-319}$, rCPE$_{37-319}$, or rCPE$_{45-319}$ fusion proteins were only briefly (5 min) incubated with BBMs (Fig. 4B). Densitometric examination of the autoradiogram shown in Fig. 4B indicated that under these experimental conditions, about twice as much large complex formed in BBMs treated with rCPE$_{37-319}$ or rCPE$_{45-319}$ fusion protein as in BBMs treated with native CPE or rCPE$_{1-319}$ fusion protein. Similar patterns of large-complex formation were observed after removal of the (His)$_6$-containing peptide from the rCPE$_{1-319}$, rCPE$_{37-319}$, and rCPE$_{45-319}$ fusion proteins (data not shown).

(iv) Cytotoxicity. A standard (16, 23, 24) ^{86}Rb release Vero cell assay for CPE cytotoxicity was used to evaluate the cytotoxic activity of *E. coli* lysates containing each of our rCPE species. The results of these experiments indicate that only lysates containing the rCPE$_{1-319}$, rCPE$_{37-319}$, or rCPE$_{45-319}$ fusion proteins (or added native CPE) displayed cytotoxic activity (Fig. 5A), even if very high (200 nM) concentrations of rCPE species containing other N-terminal and C-terminal CPE deletion fragments were added to Vero cells. Similar results (data not shown) were also observed when this assay was repeated with affinity-enriched rCPE species containing N- and C-terminal CPE deletion fragments [whether with or without the presence of the (His)$_6$-containing peptide]. All cytotoxic activity associated with rCPE$_{1-319}$, rCPE$_{37-319}$, or rCPE$_{45-319}$, whether the sample was an affinity-enriched rCPE species [with or without the (His)$_6$-containing peptide present] or an rCPE species present in *E. coli* lysates, could be specifically attributed to the presence of that rCPE species in the preparation, since (i) preincubation of these samples with anti-CPE MAb 3C9 completely abrogated the cytotoxic activity of the preparation (data not shown) while (ii) preincubation of identical samples with the nonneutralizing anti-CPE MAb 10G6 had no effect on the cytotoxicity associated with these preparations (data not shown). Quantitative analysis of the lysate cytotoxicity curves shown for the cytotoxic rCPE species in Fig. 5A indicates that the final concentration of each rCPE species causing 50% release of ^{86}Rb label was ~3.2 nM for both native CPE and rCPE$_{1-319}$ fusion protein versus only ~1.8 nM for rCPE$_{37-319}$ and rCPE$_{45-319}$ fusion proteins.

Kinetic comparisons (Fig. 5B) of the cytotoxicity of 7 nM (final concentrations) affinity-enriched preparations of these four cytotoxic rCPE species demonstrated that the rCPE$_{37-319}$ or rCPE$_{45-319}$ fusion proteins induce 50% of maximal release of the ^{86}Rb label from Vero cells after only ~4 min of treatment, while native CPE and affinity-enriched rCPE$_{1-319}$ fusion protein induce 50% release of this radiolabel only after ~11

min of treatment. Similar results were obtained with enterokinase-treated $rCPE_{1-319}$, $rCPE_{37-319}$, and $rCPE_{45-319}$ fragments (data not shown).

DISCUSSION

In the present study, a series of N- and C-terminal CPE deletion fragments have been prepared and functionally characterized to provide significant information about both the CPE structure-function relationship and CPE action.

With respect to understanding the CPE structure-function relationship, several important observations were made by using our CPE deletion fragments. First, it was shown that deleting as few as 5 amino acids from the C terminus of CPE completely abrogates receptor-binding ability. The loss of binding ability for these C-terminal deletion fragments does not appear to be attributable to gross conformational changes, since (i) our C-terminal deletion fragments retained strong reactivity with RPC-IgG (which was prepared against native CPE) under nondenaturing conditions and (ii) a conformationally sensitive monoclonal antibody (MAb 10G6 [7, 32]), also prepared against native CPE, reacted equally well with these fragments as with native CPE.

Therefore, while previous studies (6, 8) with expressed C-terminal CPE fragments have shown that such fragments can inhibit ^{125}I-CPE binding to BBMs, the inability of any C-terminal deletion fragment prepared in our present study to inhibit ^{125}I-CPE binding to BBMs offers the first direct evidence that the extreme C terminus of native CPE is actually required for receptor-binding activity. These results obtained with our C-terminal CPE deletion fragments are also consistent with the existence of a single receptor-binding site (located at the extreme C terminus) in native CPE. This finding supports previous kinetic studies (6) with a $CPE_{290-319}$ synthetic peptide suggesting that most, if not all, of the receptor-binding activity of native CPE is localized to the last 30 C-terminal amino acids of toxin. Studies are under way in our laboratory to identify the individual amino acid residues in this C-terminal region that actively mediate receptor binding.

It was also demonstrated in the present study that the first 44 amino acids can be deleted from the N terminus of native CPE without the loss of biologic activity. In fact, it was shown that, on a molar protein basis, our $rCPE_{37-319}$ and $rCPE_{45-319}$ deletion fragments actually exhibit about twice as much cytotoxic activity as does native CPE. Therefore, our present results extend previous observations (3, 4, 7) showing that limited trypsin or chymotrypsin treatment of native CPE (which remove, respectively, the first 24 and 36 amino acids from the N terminus of CPE) causes a ca. twofold activation of CPE cytotoxicity.

Considering the increased cytotoxic effects detected for our $rCPE_{45-319}$ deletion fragment, it was also interesting that in the present work, removal of only eight more amino acids from the N terminus of CPE not only eliminates this "activation effect" but also causes a complete loss of cytotoxic activity. This effect does not appear to result from gross conformational changes since, under nondenaturing conditions, our $rCPE_{53-319}$ fragment (as well as our other N-terminal CPE deletion fragments) showed equal reactivity (on a molar protein basis) with RPC-IgG to that of native CPE. Therefore, our present results suggest that some residue(s) residing between amino acids 45 and 53 of native CPE may be essential for cytotoxicity. The identity of these residues is also now being explored in our laboratory. Finally, by collating the cytotoxicity results obtained with our N- and C-terminal CPE deletion fragments, it can not be concluded that the minimum-size CPE fragment retaining cytotoxic activity would approximately encompass residues 45 to 319 of the native enterotoxin.

Our CPE deletion fragments have also provided some important information about CPE epitopes. For example, the finding that all C-terminal CPE deletion fragments prepared in this study lost reactivity with MAb 3C9 is consistent with results of previous studies (7) demonstrating that the MAb 3C9 epitope is a linear epitope located at or near the extreme C terminus of the native CPE molecule. Further, the present observation that under denaturing conditions, all C-terminal CPE deletion fragments prepared in this study exhibited ~40-fold less RPC-IgG immunoreactivity than did native enterotoxin provides the first evidence suggesting that the epitopes, including the MAb 3C9 epitope, present in the C-terminal portion of CPE represent the major linear epitopes of the native enterotoxin. Finally, since (on a molar protein basis) our C-terminal CPE deletion fragments retained ~50% of the RPC-IgG immunoreactivity of native CPE under nondenaturing conditions, the results presented in the present study also support previous MAb epitope-mapping studies (7) indicating that several conformational epitopes exist in the CPE molecule and that sequences involved in the presentation of these conformational epitopes are located throughout the CPE molecule.

Functional characterization of our N- and C-terminal CPE deletion fragments for their ability to perform the four early steps in the cytotoxic action of CPE has also contributed to our understanding of this action. For example, demonstrating in the present study that all C-terminal deletion fragments unable to bind to mammalian receptors are also noncytotoxic offers independent support for previous suggestions (25, 26) that specific binding of CPE to its receptor is the essential first step in CPE action. Similarly, since our current study did not identify any CPE deletion fragments able to form large complex but unable to perform the postbinding physical change to the CPE small complex (putative step 2 in CPE action), the present results also offer independent evidence supporting previous studies (23) of native CPE action at 4°C suggesting that large-complex formation occurs after the postbinding physical change step in CPE action. Further, by demonstrating that four different noncytotoxic N-terminal CPE deletion fragments ($rCPE_{53-319}$, $rCPE_{69-319}$, $rCPE_{103-310}$, and $rCPE_{168-319}$) could bind to receptors but were unable to undergo the postbinding physical change step in CPE action, our present results also help confirm previous suggestions (5), based on results obtained with a single $rCPE_{171-319}$ fragment, that this putative second step in CPE action is essential for cytotoxicity and that the receptor-binding and postbinding physical-change steps represent independent events in CPE action. It remains to be determined whether some residue(s) in the 8 amino acids between residues 45 and 53 of native CPE plays an active role in mediating this postbinding physical change or if this 8-amino-acid region simply maintains the proper conformation necessary for CPE to undergo this postbinding physical change.

Perhaps the most interesting results obtained during the functional characterization of our CPE deletion fragments concerned large-complex formation. First, results obtained with our CPE deletion fragments demonstrate a correlation between large-complex formation and cytotoxicity. All cytotoxic deletion fragments were able to form the large complex, whereas all noncytotoxic fragments were blocked for large-complex formation, as would be expected if large-complex formation preceded the development of cytotoxicity and was an important step in the development of cytotoxicity. Furthermore, it was also demonstrated in the present study that, on a molar basis, $rCPE_{37-319}$ and $rCPE_{45-319}$ are able to form about

twice as much large complex as is native CPE or $rCPE_{1-319}$. Since there were no apparent differences in the abilities of $rCPE_{37-319}$ or $rCPE_{45-319}$ versus native CPE or $rCPE_{1-319}$ to bind or undergo the postbinding physical-change step in CPE action, our results strongly suggest that the ca. twofold "activation" of CPE cytotoxicity noted for $rCPE_{37-319}$ and $rCPE_{45-319}$ versus native CPE or $rCPE_{1-319}$ is due to the ca. twofold increase in large-complex formation exhibited by these two N-terminal deletion fragments. Collectively, these results characterizing the ability of our N-terminal CPE deletion fragments to form the large complex are fully consistent with previous low-temperature studies (23) of native CPE action, suggesting that large-complex formation is an important step in CPE-induced cytotoxicity. Thus, our deletion fragment studies have provided important independent evidence confirming (i) the involvement of each of the three putative early steps (receptor binding, development of the postbinding physical change, and large-complex formation) in the development of CPE cytotoxicity and (ii) the order of occurrence of these three early steps.

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