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Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I

(ricin/*Shigella dysenteriae*/*Escherichia coli*/protein synthesis/ribosomes)

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ABSTRACT **_Escherichia coli_ Shiga-like toxin I, a close relative of Shiga toxin and a distant relative of the ricin family of plant toxins, inhibits eukaryotic protein synthesis by catalyzing the depurination of adenosine 4324 in 28S rRNA. By comparing the crystallographic structure of ricin with amino acids conserved between the Shiga and ricin toxin families, we identified seven potential active-site residues of Shiga-like toxin I. The structural gene encoding Shiga-like toxin I A chain (Slt-IA), the enzymatically active subunit, was engineered for high expression in *E. coli*. Oligonucleotide-directed mutagenesis of the gene for Slt-IA was used to change glutamic acid 167 to aspartic acid. As measured by an *in vitro* assay for inhibition of protein synthesis, the specific activity of mutant Slt-IA was decreased by a factor of 1000 compared to wild-type Slt-IA. Immunoblots showed that mutant and wild-type Slt-IA were synthesized as full-length proteins and were processed correctly by signal peptidase. Both proteins were equally susceptible to trypsin digestion, suggesting that the amino acid substitution did not produce a major alteration in Slt-IA conformation. We conclude that glutamic acid 167 is critical for activity of the Shiga-like toxin I A chain and may be located at the active site.**

Certain strains of *Escherichia coli* produce potent protein toxins closely resembling the classical Shiga toxin from *Shigella dysenteriae* I (1–3). These toxins, termed Shiga-like toxins (SLT), can be divided into two immunological groups: SLT-I toxins, which are neutralized by antibody against purified Shiga toxin, and SLT-II toxins, which are not neutralized by this antibody (4). SLT-I and Shiga toxin are virtually identical proteins, differing in only a single amino acid (5–7), whereas SLT-II is more distantly related, sharing 56% amino acid homology with the other two members of this family (8). Strains of *E. coli* producing large amounts of SLT-I or SLT-II have been implicated in outbreaks of neonatal and adult diarrhea (9), epidemic hemorrhagic colitis (10), and the hemolytic/uremic syndrome (11).

Toxins of the Shiga family contain a single A subunit, which is enzymically active, and multiple B subunits, which are responsible for binding holotoxin to specific receptors on the target cell surface (12). Following internalization of toxin, the A and B chains dissociate, and the A chain inhibits protein synthesis by catalytically inactivating 60S ribosomal subunits (13). We (6), and others (14), have shown that the A subunit of SLT-I (Slt-IA) shares considerable amino acid sequence homology with the A subunit of ricin, a potent plant toxin with an identical mechanism of action. Several other ribosome-inactivating proteins in plants are homologous to the ricin A chain and share a similar mechanism of action (15, 16).

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Recently a high-resolution crystallographic structure of ricin has been reported, allowing visualization of a cleft in the A subunit that may contain the enzymic active site (17). When conserved residues between the Shiga and ricin toxin families were plotted on the ricin A chain crystal structure, seven of these amino acids were found to lie in the proposed active-site cleft (Fig. 1). From this comparison, we hypothesized that one or more of these residues were likely to be important in the catalytic activity of Slt-IA. Here, we report that substitution of aspartic acid for glutamic acid 167 reduces the inhibitory activity of Slt-IA in a cell-free protein synthetic system by a factor of ≈1000.

MATERIALS AND METHODS

Construction of an Expression Vector for Slt-IA. The *slt*-IA gene was reconstructed from two previously cloned DNA fragments. By using standard techniques (18), we recovered a 650-base-pair (bp) *Hpa* II–*Hin*dIII restriction fragment from pSC2 (6) that contained the amino-terminal two-thirds of *slt*-IA and the upstream Shine–Dalgarno sequence but not the *slt*-I promoter (19). Similarly, from a subclone of pSC4 used previously for sequencing (6), we recovered a 500-bp *Hin*dIII–*Eco*RI restriction fragment that contained the carboxyl-terminal one-third of *slt*-IA and a truncated portion of *slt*-IB. These two fragments were ligated with *Acc* I–*Eco*RI-digested pUC19 (20) to reconstitute an intact *slt*-IA gene under the control of the *lacZ* promoter on pUC19 (plasmid pSC25, see Fig. 2). Strain SY327 [F⁻ *araD* Δ(*lac-pro*) *argEam rif nalA recA56*] was transformed with pSC25 and the plasmid construction was verified by restriction enzyme analysis and DNA sequencing.

Site-Directed Mutagenesis. The 1150-bp *Pst* I–*Eco*RI fragment of pSC25 was ligated into M13mp19 (21) to construct M13mp19.25. Site-directed mutagenesis of M13mp19.25 was performed with an oligonucleotide-directed mutagenesis kit as described by the manufacturer (Amersham). A synthetic oligonucleotide complementary to 5′ GTGACAGCTGATGCTTTACG 3′ was synthesized on an Applied Biosystems model 381A synthesizer and passed through a Sep-Pak C_{18} cartridge (Waters Associates). This oligonucleotide contained a single base substitution (position underlined above) to replace the GAA codon for amino acid 167 of Slt-IA, encoding glutamic acid, with GAT, encoding aspartic acid. The GAT codon for aspartic acid was selected in accordance with preferred codon usage in *E. coli* (22) and Slt-IA (6). This single base change resulted in loss of a unique *Hin*dIII restriction site within *slt*-IA that was used to identify mutant DNA in subsequent experiments. The wild-type *Pst* I-*Eco*RI fragment of pSC25 was replaced by the same fragment from mutated M13mp19.25 to generate mutant pSC25.1. This plas-

Abbreviations: SLT, Shiga-like toxin(s); Slt-IA-E167D, a mutant Shiga-like toxin I A chain with glutamic acid 167 replaced by aspartic acid.

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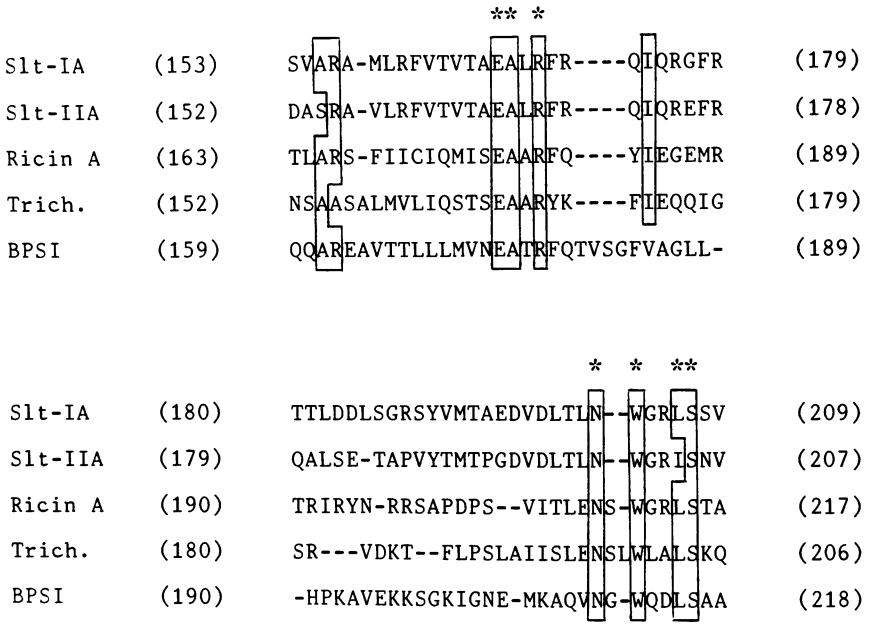

FIG. 1. Alignment of homologous amino acids in the A subunits of SLT-I (Slt-IA) and SLT-II (Slt-IIA), ricin A chain, trichosanthin (Trich.) and barley protein synthesis inhibitor II (BPSI). Conserved amino acids are enclosed in boxes. Asterisks indicate conserved residues in the cleft of the ricin A chain crystal structure. Numbers in parentheses refer to the positions of residues in the mature protein. Dashes indicate gaps introduced into the sequences to maximize alignments. The alignment of ricin A, trichosanthin, and BPSI is that presented by Ready *et al.* (16). Alignment of Slt-IA with ricin A has been presented previously (6) and the alignment of Slt-IIA is derived from that of Jackson *et al.* (8).

mid was transformed into SY327 and the construction was verified by restriction enzyme analysis and DNA sequencing.

Nucleotide Sequence Analysis. DNA was subcloned into M13mp19 and sequenced with a Sequenase kit (United States Biochemical, Cleveland, OH) and dATP[α-^{35}S] (Amersham). The universal *lacZ* primer and four synthetic oligonucleotides, spaced at 200- to 250-bp intervals along *slt*-IA, were used as primers for sequencing.

Expression of Wild-Type and Mutant Slt-IA. The expression of Slt-IA in strains of SY327 containing pSC25 (wild-type) and pSC25.1 (mutant) was compared, with strain SY327 (pUC19) serving as negative control. Cells were grown overnight at 37°C with shaking in LB medium containing ampicillin (100 μg/ml). Five OD_{600} units of cells were centrifuged at 15,000 × *g* for 5 min at 4°C. The cell pellet was resuspended in 200 μl of sample buffer (see below), boiled for 5 min, and centrifuged at 15,000 × *g* for 5 min at room temperature. The supernatant was referred to as whole cell extract and was stored at −20°C until use.

Periplasmic extracts were made from exponentially growing cells by a protocol similar to that used for extraction of Shiga toxin from *S. dysenteriae* I (23, 24). Briefly, overnight cultures were diluted 1:1000 in fresh LB medium with ampicillin (100 μg/ml) and grown to late exponential phase (OD_{600} of 0.8–1.0). Ten OD_{600} units of cells were centrifuged at 15,000 × *g* for 5 min at 4°C. The cell pellets were resuspended in 400 μl of 10 mM phosphate buffer with 140 mM NaCl (pH 7.4; PBS) containing 2 mg of polymyxin B sulfate per ml at 6000 USP units/mg (Sigma), incubated 10 min at 4°C, and centrifuged at 15,000 × *g* for 5 min at 4°C. The supernatant was referred to as periplasmic extract and was used immediately or stored at −20°C.

The proteins in whole cell and periplasmic extracts were solubilized in sample buffer and separated by electrophoresis through 12.5% polyacrylamide/sodium dodecyl sulfate gels (25). Mid-range, prestained molecular weight standards (Diversified Bioproducts, Newton Centre, MA) and purified Shiga toxin (kindly provided by A. Donohue-Rolfe, Tufts University School of Medicine, Boston, MA) were applied to each gel. Electrophoretic transfer of the separated proteins to nitrocellulose was done in transfer buffer (25 mM Tris/192 mM glycine/0.1% sodium dodecyl sulfate/20% methanol) by using a Genie electroblotting apparatus (Idea Scientific, Corvallis, OR). Immunoreactive proteins were visualized after sequential incubation with polyclonal rabbit anti-Shiga toxin antiserum (kindly provided by A. Donohue-Rolfe; ref. 26) and goat anti-rabbit immunoglobulin-conjugated alkaline phosphatase (ICN) followed by staining for phosphatase activity as described (27).

Trypsin sensitivity of wild-type and mutant Slt-IA was compared by incubation with 10-fold increasing amounts of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 37°C for 15 min. The reactions were stopped by addition of phenylmethanesulfonyl fluoride to 1 mM and the digests were solubilized in sample buffer and analyzed by electrophoresis as described above.

Assay of Protein Synthesis. Protein synthesis was assayed in a cell-free system with rabbit reticulocyte lysate and brome mosaic virus mRNA as described by the supplier (Promega Biotec, Madison, WI) except that the following quantities were added to each 50-μl reaction volume: 0.3 nmol of amino acid mixture (without methionine), 25 μCi of [^{35}S]methionine (>1000 Ci/mmol; 1 Ci = 37 GBq; Amersham), and 0.3 μg of mRNA. Reaction mixtures were incubated at 30°C for 20 min. Incorporation of radioactivity into alkali-resistant, trichloroacetic acid-precipitable material was determined as described in the Promega Biotec technical bulletin.

Periplasmic extracts were analyzed for capacity to inhibit protein synthesis by rabbit reticulocyte lysate. Prior to assay, free nucleotides and salts were removed from periplasmic extracts by gel filtration over Sephadex G-50 (Pharmacia) equilibrated in PBS (bed volume >10 times sample volume). Filtered extracts were diluted in ice-cold PBS and preincubated with reticulocyte lysate at 37°C for 30 or 60 min to inactivate ribosomes prior to addition of amino acids, [^{35}S]methionine, and mRNA. Inhibition of protein synthesis was calculated as percent of control incorporation of [^{35}S]methionine into acid-precipitable material.

RESULTS

Oligonucleotide-Directed Mutagenesis of *slt*-IA. A DNA fragment encoding intact Slt-IA was cloned into pUC19 for high expression. The construction of this plasmid, referred to as pSC25, was confirmed by restriction enzyme analysis and DNA sequencing. Oligonucleotide-directed mutagenesis was used to change the codon for glutamic acid 167 to a codon for aspartic acid, thereby generating plasmid pSC25.1. DNA sequencing of the entire *Pst* I–*Eco*RI fragment of pSC25.1 (Fig. 2) verified that the GAA codon for glutamic acid 167 had been changed to a GAT codon for aspartic acid and that no second-site mutations were created by the mutagenesis procedure. Following the nomenclature described by Knowles (28), this mutant Slt-IA was designated as Slt-IA-E167D to identify the substitution of glutamic acid 167 by aspartic acid, but it will be referred to in the text as mutant Slt-IA.

Expression of Wild-Type and Mutant Slt-IA. As shown in Fig. 3, whole cell extracts of strains with either the wild-type (lane 2) or mutant (lane 3) expression vectors contained full-length mature Slt-IA (A) as well as Slt-IA containing the signal sequence (pro-Slt-IA). In addition to these proteins, two major and several minor degradation products of Slt-IA were present; the pattern and relative amounts of these polypeptides were similar in the two strains (Fig. 3, lanes 2 and 3). In periplasmic extracts of both strains, the largest immunoreactive protein migrated with an apparent M_r of ≈32,000, identical to that of mature Shiga toxin A subunit (lane 4). Consistent with processing of Slt-IA during secretion to the periplasm (23), periplasmic extracts did not contain the higher molecular weight pro-Slt-IA. Immunoblots of serial 1:2 dilutions of extract were used to estimate the relative amounts of Slt-IA obtained from cells containing wild-type and mutant plasmids. As seen in Fig. 3, the intensity of immunoblots with undiluted mutant extract (lane 9) was intermediate between those with wild-type extracts diluted 1:2 (lane 6) and 1:4 (lane 7). From this we estimate that wild-type extract contained a 3-fold higher concentration of Slt-IA than the mutant extract. A similar ratio was observed for Slt-IA degradation products. As expected, cells containing the Slt-IA expression vectors did not produce detectable B subunit, and strain SY327, containing the control plasmid (pUC19), produced no immunoreactive material in either the whole cell or periplasmic extracts.

Inhibition of Protein Synthesis by Periplasmic Extracts. The rate of protein synthesis in our reticulocyte lysate system remained constant for >30 min after the addition of mRNA. Incorporation of radiolabel was approximately half-maximal by 20 min (data not shown), and this time point was selected

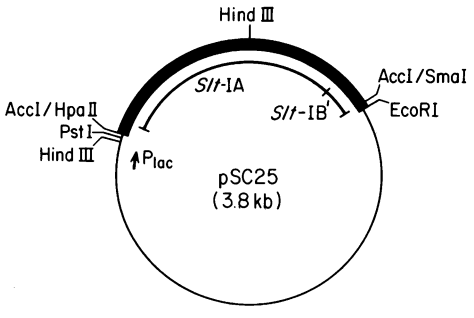

FIG. 2. Diagrammatic representation of the wild-type Slt-IA expression vector pSC25. The heavy line denotes insert DNA derived from pSC2 (*Hpa* II–*Hin*dIII) and a subclone of pSC4 (*Hin*dIII–*Eco*RI); the lighter line represents vector DNA of pUC19 (*Eco*RI–*Acc* I). Locations of the structural gene for *slt*-IA and a truncated portion of the *slt*-IB gene (*slt*-IB′) are indicated within the circle. Transcription of the *slt* genes is under the control of the *lacZ* promoter (P_{lac}) on pUC19. Locations of relevant restriction enzyme sites are indicated on the outside of the circle. kb, Kilobase pairs.

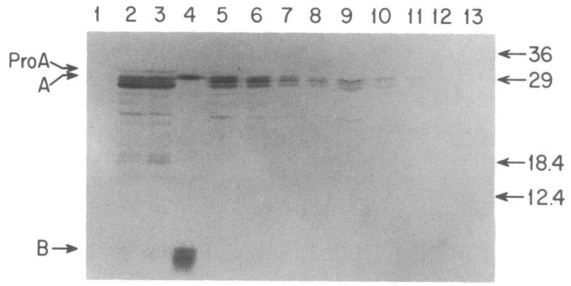

FIG. 3. Immunoblot analysis of cell extracts visualized with a colorimetric alkaline phosphatase reaction after incubation with rabbit anti-Shiga toxin antiserum and alkaline phosphatase-conjugated anti-rabbit antiserum. Samples of whole cell extracts (10 μl, lanes 1–3) and periplasmic extracts (15 μl, lanes 5–13) are shown. Identical patterns of immunoreactive material are seen with extracts of whole cells expressing wild-type (lane 2) or mutant (lane 3) Slt-IA. Both lanes contain a M_r 34,800 band representing pro-Slt-IA (ProA), a M_r 32,000 band representing mature Slt-IA (A), and several smaller degradation products. Purified Shiga toxin (1 μg, lane 4) contains mature A (A) and B (B) subunits. Serial 1:2 dilutions of periplasmic extracts from cells expressing wild-type (lanes 5–8) or mutant (lanes 9–12) Slt-IA were applied as follows: undiluted, lanes 5 and 9; 1:2, lanes 6 and 10; 1:4, lanes 7 and 11; 1:8, lanes 8 and 12. Periplasmic and whole cell extracts gave similar patterns, except that pro-Slt-IA was not seen in the former. Extracts of SY327 (pUC19) (lanes 1 and 13) contained no immunoreactive material. The positions of molecular weight standards are given as $M_r \times 10^{-3}$ at the right.

for our standard assay. Incubation of reticulocyte lysate with control extract from SY327 (pUC19) produced no inhibition of protein synthesis compared to incubations with either water or PBS, and this extract was used as the positive control (≈150,000 cpm). Negative control assays, in which mRNA was omitted, had values of ≈2000 cpm. Results with extracts containing wild-type or mutant Slt-IA are expressed as the percentage of protein synthesis obtained in the presence of control extracts assayed in parallel. Results were normalized to reflect the ≈3-fold difference in immunoreactive material between wild-type and mutant extracts.

As shown in Fig. 4, extracts containing wild-type Slt-IA were highly active, inhibiting *in vitro* protein synthesis after a 30-min preincubation with reticulocyte lysate. In contrast, extracts containing the mutant Slt-IA showed a decrease by a factor of ≈1000 in specific inhibitory activity. Preincubation of lysate with cell extracts for 60 min rather than 30 min gave similar results (data not shown). Adding a 10- or 100-fold excess of mutant extract did not affect the activity of wild-type Slt-IA, confirming that the mutant extract did not contain a spurious inhibitor of Slt-IA activity (data not shown).

Trypsin Digestion of Wild-Type and Mutant Slt-IA. A change in the susceptibility of a protein to proteolytic attack can be an indication of a change in its tertiary conformation. In an effort to show that the substitution of aspartic acid for glutamic acid at residue 167 of Slt-IA did not produce a major alteration in protein folding, extracts containing wild-type and mutant Slt-IA were incubated with increasing amounts of trypsin. As shown in Fig. 5, identical degradation patterns of these proteins resulted at each trypsin concentration, suggesting no major change in trypsin susceptibility as a result of the amino acid substitution. As previously reported (12), treatment of Shiga toxin with trypsin produced a nicked form of the A subunit (A′) with an apparent M_r of 27,500. Similar products were generated by trypsin treatment of wild-type and mutant Slt-IA (Fig. 5, lanes 5 and 9).

DISCUSSION

A number of bacterial and plant toxins act by inhibiting protein synthesis in eukaryotic cells. The Shiga and ricin

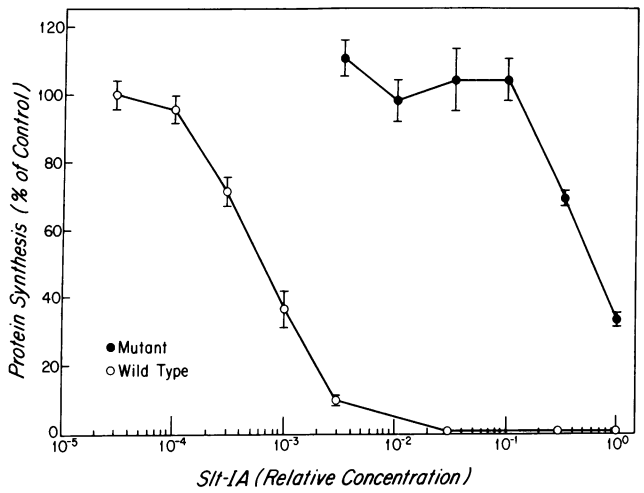

FIG. 4. Inhibition of protein synthesis by wild-type and mutant Slt-IA. Aliquots of rabbit reticulocyte lysate (35 μl) were preincubated for 30 min with various dilutions of periplasmic extracts (5 μl) containing wild-type (○) or mutant (●) Slt-IA. These lysates were then assayed for protein synthesis and compared to control lysates preincubated with extracts of SY327 (pUC19); background activity (without RNA) was subtracted from all values. Concentrations of wild-type and mutant Slt-IA were normalized for the ≈3-fold difference in immunoreactive material (Fig. 3) and plotted as relative concentration.

toxin families inhibit protein synthesis by catalytically inactivating the 60S ribosomal subunit. These toxins consist of two distinct subunits, an A subunit, which is enzymatically active after entry into the cytosol, and a B subunit, which is responsible for toxin binding to receptors on the target cell surface. Single-chain, ribosome-inactivating proteins in plants (hemitoxins), such as barley protein synthesis inhibitor II and trichosanthin, inhibit protein synthesis by a similar mechanism but are not toxic to intact cells because they lack a B subunit for binding to the cell surface.

Recent work has characterized the molecular mechanism of action of the ricin A chain. This protein catalyzes cleavage of the N-glycosidic bond in adenosine 4324 of 28S rRNA; hydrolytic removal of the adenine at this site leads to inactivation of the 60S ribosomal subunit (29). The sequence of rRNA in the vicinity of this cleavage site is highly conserved between different eukaryotic species, suggesting a key role of this site in ribosome function (29). Shiga toxin and SLT-I

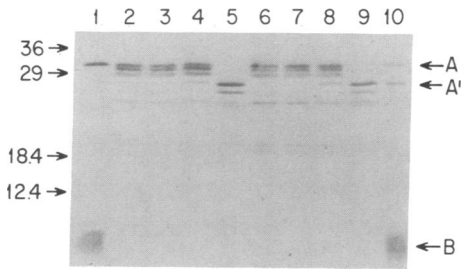

FIG. 5. Immunoblot of periplasmic extracts following trypsin digestion. Slt-IA was visualized by immunoblotting as described in the legend of Fig. 3. Periplasmic extracts (15 μl) from cells expressing wild-type (lanes 2–5) and mutant (lanes 6–9) Slt-IA were digested at 37°C for 15 min with increasing concentrations of trypsin: none, lanes 2 and 6; 0.05 μg/ml, lanes 3 and 7; 0.5 μg/ml, lanes 4 and 8; 5.0 μg/ml, lanes 5 and 9. Shiga toxin (1 μg) was included as a control in lanes 1 (undigested) and 10 (digested with trypsin, 15 μg/ml, at 37°C for 15 min). The locations of intact Shiga toxin A chain (A), the nicked form of the A chain (A'), and the B chain (B) are indicated on the right. The positions of molecular weight standards are indicated as $M_r \times 10^{-3}$ on the left.

have the same molecular mechanism of action as ricin (30), which is consistent with the previous observation that these proteins share significant amino acid sequence homology (6, 14).

The three-dimensional structure of ricin at 2.8-Å resolution reveals a prominent cleft created by the interface of three distinct A chain domains (17). Ready *et al.* (16) have suggested that amino acid residues lining this cleft and conserved within the ricin toxin family may be important in substrate binding and catalysis. As indicated in Fig. 1, 10 amino acids are highly conserved between the Shiga and ricin toxin families, and 7 of these residues lie within the major cleft in the crystal structure of the ricin A subunit.

The glutamic acid residue at position 167 was selected for alteration by site-directed mutagenesis partly because carboxylate side chains have been implicated in catalysis by various glycosyl hydrolases and transferases [e.g., lysozyme (31), sucrase-isomaltase (32)]. We chose to change glutamic acid 167 to aspartic acid because this represented a highly conservative substitution that retains the carboxyl function but alters its spatial position by ≈1 Å (28) (all other factors remaining equal). It is also noteworthy that glutamic acid side chains have been shown to be crucial for enzymic activity in another class of toxins. Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A inhibit eukaryotic protein synthesis by catalyzing the transfer of ADP-ribose from NAD to elongation factor 2 (a glycosyl transfer reaction). In both toxins it has been shown that conversion of a key glutamic acid residue, at the NAD binding site, to aspartic acid causes a >100-fold loss of ADP-ribosylation activity (33, 34).

We constructed a vector for high-level expression of Slt-IA lacking a functional B subunit (Fig. 2), so that the expressed product is not toxic to eukaryotic cells but is highly efficient in inhibition of protein synthesis *in vitro*. As shown in Fig. 4, substitution of aspartic acid for glutamic acid at position 167 in Slt-IA resulted in a reduction in the specific activity of this molecule by a factor of ≈1000 to inhibit protein synthesis *in vitro*.

Several variables that might confound interpretation of these results should be considered. As shown in Fig. 3, wild-type and mutant Slt-IA are synthesized as full-length proteins that appear to be processed correctly by signal peptidase. Wild-type and mutant Slt-IA are similarly susceptible to cleavage by trypsin (Fig. 5), providing evidence that there is no major change in conformation between the two proteins. Both A subunits undergo some proteolytic cleavage during growth of the cells, perhaps because of the absence of the B subunit, but the pattern and degree of proteolysis are similar between the two preparations. Previous experiments have demonstrated that proteolytic nicking at the carboxyl terminus of the Shiga toxin A subunit (to produce an A' subunit) results in a 6-fold increase in the activity of the subunit to inhibit protein synthesis *in vitro* (13). In our extracts, digestion with trypsin did not appear to significantly enhance inhibitory activity (data not shown), perhaps because the isolated Slt-IA chains have already undergone some proteolysis. We do not know which fragment (or fragments) of our preparations was active in inhibiting protein synthesis. However, the similar distributions of toxin-related polypeptides in the wild-type and mutant extracts make it unlikely that the 1000-fold difference in activity can be explained by differences in levels of various enzymically active species.

The large loss in specific activity of Slt-IA following a single conservative amino acid substitution for glutamic acid 167, a residue conserved across the ricin and Shiga toxin families and which lies in a cleft in the crystallographic structure of the ricin A chain, suggests that this residue may be part of the active site of these toxic molecules. Experi-

ments to examine the effect of replacing the homologous glutamic acid residue in the ricin A chain will be of interest.

Note Added in Proof. After submission of this article, it came to our attention that Kozlov *et al.* (35) have similarly reported amino acid homology between the A subunit of Shiga toxin and ricin.

We gratefully acknowledge Michael P. Ready *et al.* (16) and Nancy A. Strockbine *et al.* (7) for providing us with manuscripts prior to publication and Arthur Donohue-Rolfe for kindly providing us with purified Shiga toxin and anti-Shiga toxin antiserum. This work was supported by grants from the National Institutes of Health (AI22021, AI22848, and AI18045). Partial support was also received from the Shipley Institute of Medicine. C.J.H. is recipient of a National Research Service Award from the National Institute of Allergy and Infectious Diseases (AI07663) and S.B.C. is recipient of an Upjohn Scholar Award.

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